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BIOLOGICAL BULLETIN

PUBLISHED BY
THE MARINE BIOLOGICAL LABORATORY

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CONTENTS

No. 1. February, 1937

PAGE

von Brand, Theodor
Observations upon the Nitrogen of the Particulate Matter in the Sea 1
Dethier, Vincent G.
Gustation and Olfaction in Lepidopterous Larvae 7
Kleinholz, L. H.
Studies in the Pigmentary System of Crustacea. I. Color changes and diurnal rhythm in Ligia baudiniana 24
Cameron, John Andrew
The Mitotic Rate in Tadpole Skin after Repeated Injury 37
Arey, Leslie B.
Observations on Two Types of Respiration in Onchidium 41
Clancy, C. W., and G. W. Beadle
Ovary Transplants in Drosophila melanogaster: Studies of the Characters Singed, Fused, and Female-Sterile 47
Welsh, J. H., and F. A. Chace, Jr.
Eyes of Deep Sea Crustaceans. I. Acanthephyridae 57
Guttman, S. A.
Evidence for the Production of Accelerator and Depressor Substances by Ultraviolet Radiation of Limulus Muscle 75
Odlaug, Theron O.
Notes on the Development of Gorgodera amplicava in the Final Host 80
Morgan, T. H.
The Behavior of the Maturation Spindles in Polar Fragments of Eggs of Ilyanassa Obtained by Centrifuging 88
Gilchrist, Francis G.
Budding and Locomotion in the Scyphistomas of Aurelia 99
Dawson, J. A., Walter R. Kessler and Joseph K. Silberstein
Mitosis in Amoeba proteus 125

No. 2. April, 1937

Turner, C. L.
Reproductive Cycles and Superfetation in Poeciliid Fishes . . 145

PAGE

VON BRAND, THEODOR, NORRIS W. RAKESTRAW AND CHARLES E. RENN
The Experimental Decomposition and Regeneration of Nitrogenous Organic Matter in Sea Water 165
KLEINHOLZ, L. H.
Studies in the Pigmentary System of Crustacea. II. Diurnal movements of the retinal pigments of Bermudan decapods . . . 176
RENN, CHARLES E.
Bacteria and the Phosphorus Cycle in the Sea 190
SONNEBORN, T. M.
Induction of Endomixis in Paramecium aurelia 196
HODGE, CHARLES, 4TH
Some Effects of Diet on the Gastric Epithelial Cells of the Grasshopper, Melanoplus differentialis Thomas 203
FAURÉ-FREMIET, E.
Licnophora lyngbycola, a New Species of Infusorian from Woods Hole 212
ALLEE, W. C., AND GERTRUDE EVANS
Some Effects of Numbers Present on the Rate of Cleavage and Early Development in Arbacia 217
FULLER, JOHN L.
Feeding Rate of Calanus finmarchicus in relation to Environmental Conditions 233
GRAFFLIN, ALLAN L.
Cyst Formation in the Glomerular Tufts of Certain Fish Kidneys 247

NO. 3. JUNE, 1937

SHULL, A. FRANKLIN
The Production of Intermediate-winged Aphids with Special Reference to the Problem of Embryonic Determination 259
PARKER, G. H.
The Loping of Land-Snails 287
HASLER, ARTHUR D.
The Physiology of Digestion in Plankton Crustacea. II. Further studies on the digestive enzymes of (A) Daphnia and Polyphemus. (B) Diaptomus and Calanus 290
COONFIELD, B. R.
Symmetry and Regulation in Mnemiopsis leidyi, Agassiz 299
DALCQ, A., AND G. VANDEBROECK
On the Significance of the Polar Spot in Ripe Unfertilized and in Fertilized Ascidian Eggs 311

PAGE

HYMAN, LIBBIE H.
Reproductive System and Copulation in Amphiscolops langerhansi (Turbellaria Acoela) 319
MILES, SAMUEL STOCKTON
A New Genus of Hydroid and its Method of Asexual Reproduction 327
HOPKINS, DWIGHT LUCIAN
The Relation between Food, the Rate of Locomotion and Reproduction in the Marine Amoeba, Flabellula mira 334
ABRAMOWITZ, ALEXANDER A.
The Chromatophorotropic Hormone of the Crustacea 344
SCHECHTER, VICTOR
Calcium Reduction and the Prolongation of Life in the Egg Cells of Arbacia punctulata 366
CHASE, HYMAN Y.
The Effect of Ultra-violet Light upon Early Development in Eggs of Urechis caupo 377
CHURNEY, LEON AND HERBERT M. KLEIN
The Electrical Charge on Nuclear Constituents (Salivary Gland Cells of Sciara coprophila) 384
LOOSANOFF, VICTOR L.
Development of the Primary Gonad and Sexual Phases in Venus mercenaria Linnaeus 389
LOOSANOFF, VICTOR L.
Seasonal Gonadal Changes of Adult Clams, Venus mercenaria (L.) 406
FOX, DENIS L., H. U. SVERDRUP AND JOHN P. CUNNINGHAM
The Rate of Water Propulsion by the California Mussel 417
INDEX 439

Vol. LXXII, No. 1 February, 1937

THE

BIOLOGICAL BULLETIN

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OBSERVATIONS UPON THE NITROGEN OF THE PARTICULATE MATTER IN THE SEA [1]

THEODOR VON BRAND

(From the Woods Hole Oceanographic Institution)

The present investigation has been undertaken in order to secure data on the amount of organic matter present in particulate form in different regions of the sea. Such information is of importance because in the cycle of life in the sea a very large part of the living matter must occur as nanoplankton. These organisms, together with the more resistant portions of decomposing organisms, detritus, make up the particulate matter which has been studied. Data have accumulated rapidly concerning the dissolved substances such as nitrate, phosphate, etc. which appear in the later stages of the cycle of decomposition but at present almost nothing is known of the quantitative occurrence of these materials while they are bound up in organized matter.

For practical analytical reasons, as well as because of the supreme importance of nitrogenous compounds in the biological cycle, the distribution of particulate matter has been measured in terms of the nitrogen content. Water samples of one to four liters were collected with Nansen bottles and preserved by the addition of 20 cc. of formalin per liter. The particulate matter was concentrated according to the centrifugation method of Steemann-Nielsen and von Brand (1934). The nitrogen content was determined according to von Brand's (1935) modification of Krogh and Keys' (1934) method. In every case duplicate analyses were performed. With a few exceptions, in the case of very low values, they agreed in the range of 10 to 15 per cent. All organisms of about the size of copepods or above were removed. The values obtained thus represent the nitrogen content of the nanoplankton and detritus.

Throughout the whole procedure every care was taken in order to avoid contamination with nitrogen-containing substances. There were no indications of any great amount of contamination, but the possibility of a certain degree of contamination cannot be excluded with certainty (for example, from the wire or the closing mechanism of the Nansen bottles). It is not impossible, on the other hand, that during the concentration a certain amount of the particulate matter was lost. The method of concentration gave very satisfactory results with nordic plankton (Steemann-Nielsen and von Brand, 1934); but with Mediterranean plankton considerable losses were reported (Bernard and Fage, 1936). This may, however, be due to slight modifications of technique. Dr. John Fuller (personal communication) tested the method with a diluted culture of *Nitschia closterium.* He compared the values obtained after centrifugation with those calculated from previous direct counts on the undiluted culture. He recovered 94 per cent, whereas Bernard and Fage lost 50 per cent of *Nitschia longissima.*

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[1] Contribution No. 123 of the Woods Hole Oceanographic Institution.

In Table I are summarized the analytical data from which the results given in Table IV are derived. They demonstrate the order of accuracy which can be expected from the nitrogen method. The percentage error is about the same in most of the data reported in the other tables. The N values actually determined were also in most other cases approximately as high as those shown in Table I. In cases where only small amounts of nitrogen were expected, greater amounts of water were centrifuged (for the single analysis of the deep sea samples usually 1,000–2,000 ml., for those of the Gulf of Maine 200–400 ml.). In some analyses of the deep sea samples with nitrogen values below 1γ N

TABLE 1

Accuracy of the nitrogen method

Water centrifuged for the single analysis in milliliters	Naphthyl red in milliliters						Naphthyl red in milliliters corresp. to 1γ N	γN in particulate matter	
	I. Analysis			II. Analysis				I. Analysis	II. Analysis
	Control precip.	Organ. precip.	Diff.	Control precip.	Organ. precip.	Diff.			
200	2.88	1.30	1.58	2.70	1.00	1.40	0.45	3.5	3.1
100	3.40	2.45	0.95	3.39	2.19	1.20	0.44	2.2	2.7
200	3.27	1.83	1.44	3.31	1.63	1.68	0.44	3.3	3.7
100	2.75	1.34	1.41	2.75	1.46	1.29	0.47	3.0	2.7
100	3.24	1.08	2.16	3.00	0.50	2.50	0.50	4.3	5.0
100	3.18	1.59	1.59	2.96	1.46	1.50	0.45	3.5	3.2
200	3.23	0.65	2.58	3.17	0.32	2.85	0.45	5.7	6.3
200	3.03	0.34	2.69	2.73	0.30	2.43	0.44	6.1	5.5

in the single analysis the percentage error was appreciably higher. The greatest percentage difference observed between two analyses of the same water sample occurred in the 1,300-meter sample of the Sargasso Sea station: I. analysis 0.40γ N, II. analysis 0.85γ N.

Confidence in the quantitative adequacy of the methods employed is also given by a comparison of the results obtained at mid depths in the Gulf of Maine (Table V) with similar estimates of the phosphorus present in particulate form in these waters. Analyses made by Dr. Homer Pyne Smith indicate that about 5γ per liter of PO_4 is present throughout the year in a form which can be separated by filtration. This is one-third the quantity of nitrogen found in particulate form; exactly the ratio to be expected in particles of organic origin.

RESULTS

Table II shows the vertical distribution of the nitrogen in the particulate matter in the Sargasso Sea. The relatively low surface value of 8.4γ per liter is in accordance with the scarcity of plankton in this part of the Atlantic. The amount of nitrogen decreases with depth, first rapidly, then more slowly until it is only a fraction of 1γ per liter between 1,100 and 1,300 meters depth. It is apparent that in the deeper layer the values are again somewhat higher. Between 1,400 and 3,000 meters they vary from .5 to 2.8γ per liter. In the deepest sample the nitrogen content was so low that it could not be determined in the particulate matter of the 1.8 liter of water available. The determinations demonstrate that at least in the upper 3,000 meters everywhere particulate organic substance is in suspension. Of course, it cannot be stated that it represents the amount of living substance. It is reasonable to

TABLE II

Vertical distribution of the nitrogen of the particulate matter in the Sargasso Sea.
Atlantis Station 2639. Latitude N. 35° 08′. Longitude W. 66° 30′.
July 10–11, 1936.

Depth in meters	Nitrogen γ per liter	Depth in meters	Nitrogen γ per liter	Depth in meters	Nitrogen γ per liter	Depth in meters	Nitrogen γ per liter
0	8.4	500	1.4	1,100	.2	2,000	1.4
50	5.0	600	0	1,200	.2	2,250	2.8
100	3.4	700	.7	1,300	.3	2,500	.5
200	2.3	800	1.1	1,400	1.6	2,750	.8
300	2.0	900	.8	1,600	1.7	3,000	2.3
400	1.4	1,000	1.6	1,800	2.9	4,500–4,750	0

expect that at least a part is due to the residues of dead organisms or to solid excreta.

A similar series of determinations was secured from the water on the outer slope of Georges Bank (Table III). The distribution of nitrogen, which shows a progressive decrease from the surface downward to 300 meters, is similar to that occurring at comparable depths in the Sargasso Sea. In the coastal water the nitrogen values are about twice as high as in mid-Atlantic at 50 to 300 meters, and the contrast is even greater at the surface.

TABLE III

Vertical distribution of the nitrogen of the particulate matter in the offshore waters.
Latitude N. 40° 20′, Longitude W. 67° 39′. July 24, 1936.
Depth of station, 1,000 meters.

Depths in meters	Nitrogen γ per liter
0	29
50	11
100	7
200	4.7
300	3.6

A series of determinations has been carried out on surface samples from the region of Georges Bank in order to study the variability in a small area (Table IV). With the exception of the very high value of 47γ N per liter, all figures lie within the range of 18–34γ N per liter.

TABLE IV

Horizontal distribution of the nitrogen of the particulate matter of surface water near Georges Bank.

Date	Position		Nitrogen γ per liter
	Latitude N.	Longitude W.	
July 21, 1936	40° 07′	69° 02′	18
July 21, 1936	40° 09′	69° 04′	24
July 23, 1936	40° 16′	68° 08′	18
July 24, 1936	40° 20′	67° 39′	29
July 25, 1936	41° 21′	66° 07′	47
July 23, 1936	41° 22′	66° 08′	34
July 23, 1936	40° 22′	67° 52′	30
July 26, 1936	40° 27′	67° 42′	29

In the Gulf of Maine water was studied from three deep stations and one shallow station. The results are summarized in Table V. The distribution of the particulate matter in the upper 40 meters was irregular, but fell very nearly in the range of the values from the surface water near Georges Bank. It is reasonable to assume that the differences found are due to an irregular distribution of the phytoplankton (*cf.* Gran, 1933; Gran and Braarud, 1935). Below 40 meters the conditions in the three deeper stations are on the whole similar. We find at intermediate depths a relatively small amount of particulate matter, whereas in the deeper layers the amount is always again higher. It should be noticed that both the minima and maxima are within very narrow limits the same at the three stations (9–10 and 16–17γ nitrogen per liter respectively). The differences in the depths at which the minimum and maximum concentrations of nitrogenous particulate matter occur are doubtless due to hydrographic as well as biological causes. Much more information is required before they can profitably be discussed. The striking feature of the waters of the Gulf of Maine in contrast to those from offshore is the higher and more uniform concentration of the particulate matter in the subsurface layers. May this not

TABLE V

Vertical distribution of the nitrogen of the particulate matter in the Gulf of Maine. Depths not corrected for wire angle.

	Atlantis Station No.			
	2642	2644	2643	2654
Date.......	Aug. 8, 1936	Aug. 18, 1936	Aug. 10, 1936	Aug. 19, 1936
Position....	40° 46′ N. 71° 31′ W.	41° 53′ N. 69° 24′ W.	42° 19′ N. 69° 17′ W.	42° 59′ N. 70° 12′ W.
Depth of station...	64 meters	ca. 200 meters	229 meters	ca. 160 meters
Depth of sample. m.	Nitrogen in γ per liter	Nitrogen in γ per liter	Nitrogen in γ per liter	Nitrogen in γ per liter
0	23	22	12	34
20	25	19	—	29
40	20	28	15	12
60		11	11	10
80		13	10	13
100		11	10	17
120		10	11	—
140		10	17	14
160		11	17	15
180		16	14	
200		16	14	
215			15	

be explained by the consideration that in a coastal basin particulate matter cannot sink out of reach of the tidal currents, which extend even to the bottom, and is thus kept more uniformly in suspension?

I should like to acknowledge my indebtedness to Dr. A. C. Redfield, Mr. H. R. Seiwell, Mr. H. C. Stetson and Dr. E. E. Watson for having collected the water samples and to Dr. N. W. Rakestraw for having prepared some of the reagents. The work was done during the tenure of a fellowship granted by the Woods Hole Oceanographic Institution.

SUMMARY

The distribution of nitrogen contained in the particulate matter suspended in the sea has been studied in the Sargasso Sea, on the outer slope of Georges Bank, and in the Gulf of Maine. In the oceanic water the concentration of nitrogenous particulate matter declines sharply with depth to 500 meters and tends to increase slightly below 1,400 meters. On the outer slope of Georges Bank the distribution is similar down to 300 meters, but the concentrations are about twice as great as in mid-ocean at comparable depths. In the Gulf of Maine the nitrogenous particulate matter is more uniformly distributed with respect to depth and is present in greater amounts in the subsurface water than on the outer slope of Georges Bank.

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GUSTATION AND OLFACTION IN LEPIDOPTEROUS LARVÆ

VINCENT G. DETHIER

(From the Biological Laboratories, Harvard University, Cambridge, Massachusetts)

INTRODUCTION

Investigations into the nature of the chemical senses of lepidopterous larvæ have been few. McIndoo (1919) demonstrated the existence of an olfactory sense, but no one has made a special study of the gustatory sense. It is the purpose of this paper to present data on the gustatory thresholds for sucrose, dextrose, and hydrochloric acid and data on the olfactory threshold for ethyl alcohol; to locate and describe the sense organs involved; and to show what part these two senses play in the selection of food plants.

I wish to express my sincere thanks to Professor C. T. Brues and Professor T. J. B. Stier for their help and criticism during the course of this investigation.

Gipsy moth caterpillars (*Porthetria dispar*) were employed as suitable test subjects for the determination of the taste threshold of various substances.

Each insect was tested individually and the gastronomic history of each followed. One which would accept water was eliminated because this was taken as a sign that the animal would accept indiscriminately any liquid offered to it. If the water was refused, drops of sucrose in dilutions of .0001M, .001M, .01M, .1M, .5M and 1M were offered in succession and the point noted where the larva accepted the drop.

From this it appears that *P. dispar* larvæ can taste sucrose solutions as dilute as .1M. This threshold varies slightly with individuals as some respond only to .5M solutions. The threshold appears to be independent of the temperature changes occurring in the natural environment of the larvæ and independent of larval age. Fatigue of the organs of taste, if it occurs, must be negligible as no indication of this was observed.

By presenting the solutions in alternation with water and at variable strengths, it was shown that most larvæ could distinguish between .1M and .5M.

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When .1M was offered following a dose of water, it was accepted. When .1M was presented after a dose of .5M, it was refused. Thus we conclude that larvæ can distinguish between .1M and .5M sucrose solutions. Similar experiments proved that the larvæ can also distinguish .5M from 1M and 1M from 2M. The readiness with which larvæ accepted and the length of time they imbibed might also be used as an index of their preferences.

The threshold for dextrose solutions was determined in much the same manner as that for sucrose. Larvæ could distinguish concentrations of dextrose as low as .5M. For a determination of a hydrochloric acid threshold the fundamental procedure was altered in one respect only. Larvæ would accept hydrochloric acid solutions below a certain concentration. In order to determine, therefore, what the lowest acid concentration was which larvæ could detect, it was necessary to use as a control some solution which was readily accepted. For this reason only those larvæ were tested which accepted water. Taking the most dilute solution which the larvæ *refused* as the threshold concentration, this was found to be .05M and possibly as low as .005M.

As larvæ of other species became available, similar experiments were repeated on them and the thresholds of the following species were found to be the same as those of *P. dispar: Anosia plexippus, Papilio polyxenes, Diacrisia virginica, Estigmene acræa, Samia cecropia, Isia isabella, Euchætias egle,* Hesperiidæ (sp. ?), Noctuidæ (sp. ?), and a Black Woolly Bear caterpillar. The HCl threshold of *Anosia plexippus* was definitely .005M.

It is important to note that the threshold concentrations in man are approximately eight times more dilute than those of caterpillars. The importance of this comparison is apparent when we discuss the experiments on plant choice.

It is an exceedingly difficult matter to determine the threshold for olfaction. Numerous olfactometers have been designed for insects (McIndoo, 1927; Ripley and Hepburn, 1929; and Wirth, 1928), but none of these can be used with caterpillars. Some are designed for the use of different kinds of odors rather than different concentrations of the same odor; others, for winged insects only; and all, for adult insects only. After working with a great many caterpillars I have come to the conclusion that they possess a short-range olfactory sense. On account of this all olfactometers so far designed are inadequate since the source of the odor is distant from the chamber containing the animals, and by the time the odor reaches the larvæ it is too dilute to be detected.

The most successful method was adapted from that used by Parker and Stabler (1913) for human beings. It consisted, in short, of evaporating a known weight of ethyl alcohol in a known quantity of air. Before the experiment the container was cleaned thoroughly and rid of all odor (as far as the experimenter could determine). Four-hundredths of a cc. of 10M alcohol (460.0 gm. of alcohol to 1000.0 cc. of water) was placed in the glass jar which was then sealed, inverted, and placed in the sunlight. The jar was shaken frequently. Twelve hours were judged adequate time for complete evaporation and nearly complete diffusion. At this time one *Euchœtias egle* larva reposing on an odorless strip of paper was quickly thrust into the jar. Every attempt was made to prevent the air mixture from becoming too greatly disturbed. The time necessary for a response was usually three to four minutes. The best responses occurred when the larvæ were more or less motionless.

The typical response to an odor, particularly to a disagreeable one, consists of characteristic movements of the mouthparts. The labrum is retracted; the labium, extended forward and downward. The maxillæ, since they are more or less fused to the lower lip, of necessity follow a like course. The mandibles open, and then the whole process is reversed, beginning with the closing of the mandibles. A rapid alternation of this "spitting" motion often accompanied by a violent trembling of the antennæ and maxillæ constitutes a perfect response to an odor.

The threshold was taken as the lowest concentration which would cause a response. Control experiments were run using one drop of water under similar conditions. The threshold of taste is so very high that all odors used in this experiment were below the threshold of taste; therefore, responses were truly due to olfaction.

Parker and Stabler computed the threshold concentration of ethyl alcohol for man as 5.750 mg. ethyl alcohol/liter of air. Wirth arrives at an ethyl alcohol threshold concentration of 5–20 mg./liter for an ichneumonid. On the basis of similar calculations I find the threshold of *E. egle* to be 55 mg./liter. This is roughly four times more than that of the ichneumonid and 9.5 times more than that of man, while that for the ichneumonid is 1.5 times more than that of man.

It seemed obvious from the beginning that the caterpillar's organs of taste were located in the head and a series of experiments was begun in an attempt to locate these organs. Two procedures were employed: either to stimulate areas locally and so determine the seat of taste reception, or to remove appendages and areas of the head capsule one by one or in various combinations until a point was reached where the larva no longer responded to taste. A sharp lookout was kept for any rise in the threshold as various areas were removed. Larvæ of *I. isabella* and *E. egle* were used (*cf.* Fig. 1).

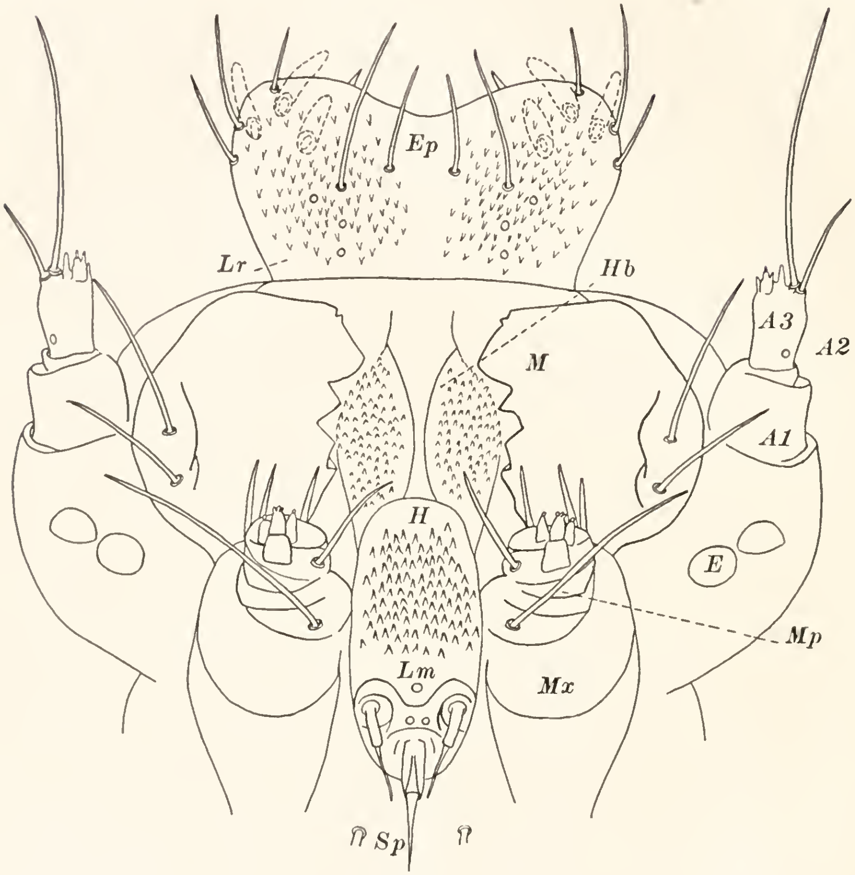

FIG. 1. Ventral view of the mouthparts of the larva of *Isia isabella*. *Lr*, labrum; *Ep*, epipharynx; *Hb*, hypopharyngeal lobe; *M*, mandible; A1, A2, A3, first, second and third segments of the antenna; *E*, eye; *H*, hypopharynx; *Lm*, labium; *Mx*, maxilla; *Mp*, maxillary palpus; *Sp*, spinneret.

When a larva was to be stimulated locally, it was tied down on its back to a piece of cardboard by means of two loops of string, one around the neck, the other around the sixth or seventh abdominal segment. Each loop passed over the caterpillar and then through two holes in the cardboard to the bottom side where the two ends were tightly tied. This "operation board" was then strapped to the stage of a binocular dissecting microscope by means of two rubber bands. A fine needle was used to bring a drop of solution to the mouthparts.

After a study of the types of responses due to a dry needle or to plain water had been made, it was possible to distinguish no response from a positive response (to a test solution) on the basis of the following criteria:

1. Response to a mechanical stimulus may or may not occur as a single motion of the organ away from the source of stimulus.
2. Response to water is either neutral or passive, i.e., no response or a feeble imbibing. If the larva has previously had its fill of water, the latter does not occur.
3. Response to sweet solutions consists of a greedy drinking.
4. Response to a salt, sour, or bitter solution consists of a decisive attempt to turn away and of a vigorous spitting motion of the mouthparts.

TABLE I

Local stimulation of head appendages. Minus sign signifies a negative response and plus sign signifies a positive response.

Solution	Maxilla	Epipharynx	Hypopharynx	Antenna
HCl (.05M)	−	+	+	−
H_2O	−	−	−	−
Sucrose (1M)	−	+	+	−

After treatment with a solution the mouthparts were rinsed with water and dried with a bit of paper towel, and the test was not repeated until the larva had quieted down and become motionless. An examination of Table I will show the areas stimulated, the solutions employed, and the results observed.

The next procedure consisted in removing various areas. This was accomplished by means of an electric cautery needle in some cases and by amputation with forceps in others. In either case the wounds closed satisfactorily, and the shock of the operation passed in from fifteen to thirty minutes. As an extra precaution, however, no test was made until a recovery period of twenty-four hours had elapsed. Following this interval tests were made in the usual manner.

It was found that taste could be abolished entirely only by removing both the epipharynx and hypopharynx. The removal of either one caused the responses to be weaker but did not raise the threshold; removal of any of the other appendages (antennæ included) either singly or in combination produced no effect whatsoever. These experiments indicated that the organs of taste are located in the epipharynx and hypopharynx.

In order to check the conclusions deduced from the above experiments, namely, that organs of taste are located primarily, if not entirely, in the epipharynx and hypopharynx, a histological examination was made of the labrum and labium of first instar tent caterpillars (*Malacosoma americana*) and of last instar flour moth larvæ (*Ephestia kuehniella*).

The various types of innervated organs found on these two appendages are listed in Table II.

Table II

Sense organs of the epipharynx and hypopharynx.

Epipharynx	Hypopharynx
Sensilla placodea	Large spines
Sensilla trichodea	
Epipharyngeal setæ	
Sinus organs	
Large spines	
Small spines	Small spines

I have not been able to find any account of the organs present on the hypopharynx of caterpillars. The organs occurring ventrally on the epipharynx have been studied externally in *Bombyx mori* (Grandi, 1923) and externally and histologically in *Orthosia lota* (Henig, 1931). I have studied the external anatomy and histology of the same organs in *Malacosoma americana, Ephestia kuehniella, Isia isabella, Euchætias egle,* and *Protoparce quinquemaculata.* The number and location of these organs is nearly identical in all of these species.

Sensilla placodea.—These are thin flat circular plates in the integument. There is no aperture present. Each is innervated by a single bipolar sensory cell. These "sense domes" (Henig) or "pores" (McIndoo) occur in two groups of three on either side of the median line.

Sensilla trichodea.—Typical hairs are found universally over the bodies of caterpillars. Usually from ten to fourteen are distributed over the ventral surface of the labrum.

Epipharyngeal Setæ.—These occur in two groups of three on the anterior edge of the labrum to either side of the median line. They are wide, flat, hollow organs which do not usually articulate in a socket.

Sinus Organs.—Henig (1931) seems to have been one of the first to mention these two strange organs occurring close to the ventral surface of the posterior part of the labrum. The sinus organs (*cf.* Fig. 2) are marked by no external differentiation of the chitin. In the species which I examined these organs occurred as round diffuse bodies at the end of a rather prominent nerve trunk. They are approximately twenty micra thick, staining dark blue with Mallory's triple connective tissue stain and dark red with Delafield's hematoxylin and eosin.

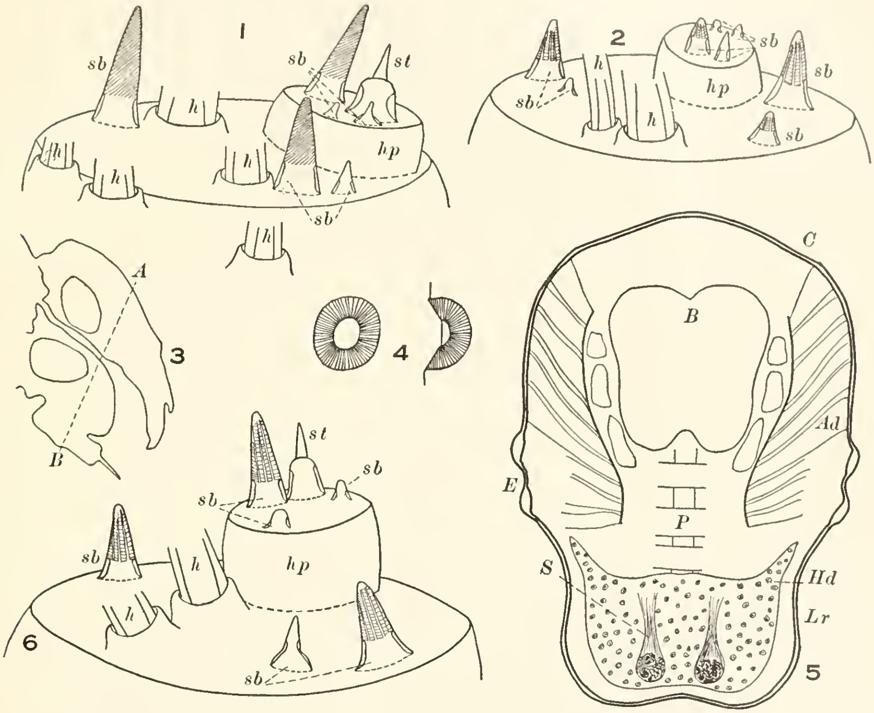

FIG. 2. 1, Terminal end of the third segment of the antenna of an *A. plexippus* larva. 2, End of the third segment of the antenna of a *S. cecropia* larva. 3, Diagram of a longitudinal section through a larval head showing where the part of the head anterior to the line *A–B* was removed. 4, Surface and profile views of a typical pore. 5, Section through the head of *M. americana* parallel to the face showing the sinus organs. 6, End of the third segment of the antenna of a *P. quinquemaculata* larva. *h,* hair; *sb,* sensillum basiconicum; *st,* sensillum styloconicum; *hp,* headpiece; *C,* cuticula; *B,* brain; *E,* eye; *Ad,* adductor muscles; *P,* pharyngeal muscles; *S,* sinus organ; *Hd,* hypodermis; *Lr,* labrum.

Spines.—Both large and small spines occur rather generally over the surfaces of the epipharynx and hypopharynx. The two types differ in size and thickness of wall only, but both are so small that it is difficult to determine much concerning their structure. They appear to be hollow and project up from the even surface of the surrounding chitin, i.e., they are neither raised on bases nor set in sockets. All of them appear to be innervated, but Henig (1931) did not describe them.

We see from this list that there is an abundance of sensilla in which the sense of taste might be located. One fact, however, throws out a huge number of possible organs, namely, that caterpillars with the labrum removed can still taste. Obviously there must be organs of taste in the hypopharynx and only large and small spines occur here. Of all the organs in this list the hairs are decidedly tactile; the epipharyngeal setæ, rather too thick-walled to be gustatory in function; the "pores," too widely distributed to be gustatory (they also occur universally over the body and its appendages). The sinus organs offer a tempting possibility. Since only spines occur on the hypopharynx, it seems as though we should attribute the perception of gustatory stimuli to them. I do this with reservations because I rather favor the hypothesis that the function of these spines is to prevent food from slipping within the mouth. Future study of the structure of the sinus organs will reveal whether or not they are adapted for receiving gustatory stimuli.

It was observed in repeated experiments that larvæ responded to odors only when the source of the odor was near the head of the animal. The close proximity of odors to any portion of the body except the head brought no response. When a larva was cut in half, only the anterior portion responded to the odor. If the animal was severed between the thorax and the abdomen, the same was true. The isolated head responded vigorously to odors; the remainder of the body did not. If merely the face of the larva was removed along the line *A–B* (*cf.* Fig. 2), there was still no response to odors. (In this case since the mouthparts were removed, a typical response would have been simply a turning away from the source of the odor.) This would seem to indicate that the organs of olfaction were localized in that portion of the head anterior to the line *A–B*.

As had been done in the case of taste, the olfactory responses were determined after the removal of various appendages by the electric cautery or by fine forceps.

A twenty-four-hour period elapsed between the time of operation and the time of the test. In separate experiments the labrum, hypopharynx, these two together; the antennæ, maxillæ and these two together, were removed. Only when both the antennæ and maxillæ were removed was the response absent; in all other cases olfaction was not at all impaired. The following species were tested: *Porthetria dispar, Isia isabella, Anosia plexippus, Euchætias egle, Samia cecropia, Pieris rapæ, Papilio polyxenes, Papilio turnus, Ephestia kuehniella,* and two noctuids.

The location of the seat of smell was further determined in greater detail by removing the parts as shown in Table III.

These experiments indicate that the seat of olfaction lies in the terminal segments of the antennæ and maxillæ. It now remains to be seen whether these segments bear sensilla that could possibly be olfactory in function.

TABLE III

Olfactory responses after removal of various appendages.

Parts removed	Responses observed
a. Segments I, II, + III of the maxillæ with Segment III of the antennæ	None
b. Segments I, II, + III of the antennæ with Segment III of the maxillæ	None
c. Segment III of both antennæ and maxillæ	None

The antennæ of caterpillars are located laterad of the bases of the mandibles, usually nearer their anterior articulations. It is generally agreed that the antenna has three segments. Segment I (basal), large and membraneous, contains no sense organs. Segment II is reduced to a mere ring distinguished solely by four sensilla placodea. Segment III (*cf.* Fig. 2) is characterized by a galaxy of sensilla differing in structure, number and distribution with each species.

I have examined a wide variety of species including representatives of Noctuidæ, Sphingidæ, Arctiïdæ, Lasiocampidæ, Saturniïdæ, Hesperiidæ, and Danaidæ. A generalized outline of sensilla occurring on the terminal segment follows:

1. Two large hairs (sensilla trichodea), one of which is approximately four times longer than the other.

2. A palpus-like headpiece.

3. Four sensilla located on the headpiece, two of which are blunt or acute cones (sensilla basiconica) either appreciable in size or barely discernible projections of the chitin, one an acute peg mounted on a conical base (sensillum styloconicum), and finally a rather large cone (sensillum basiconicum).

4. Two large cones (sensilla basiconica) located to either side of the headpiece.

5. Two small rather inconspicuous cones (sensilla basiconica) located near the base of the headpiece.

6. A pore plate (sensillum placodeum) situated almost at the basal extremity of the terminal segment.

In some species examined the three large sensilla basiconica were found to be characteristically sculptured (*cf.* Fig. 2). One type appears as spiral ridges on the outside of the cone while the other type exists as internal ridges running from top to bottom and connected by fine cross striations. The purpose of these ridges and striations is not known, nor have they ever been reported in the literature. Characteristic of all these organs (hairs excepted) is the fact that they are hollow and their walls except at the bases are less than one micron in thickness.

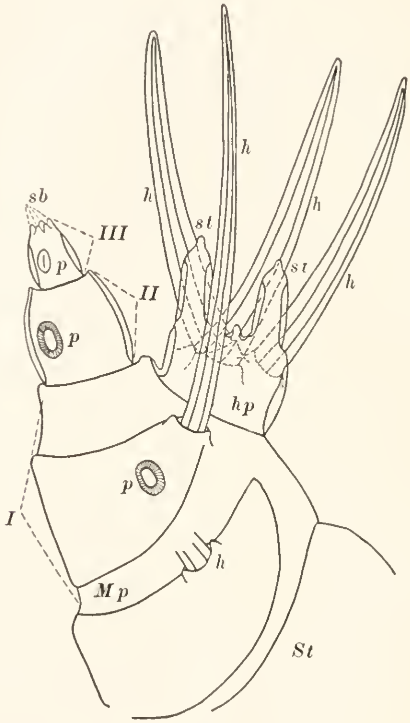

FIG. 3. Maxilla of a *P. quinquemaculata* larva. *h*, hair; *st*, sensillum styloconicum; *hp*, headpiece; *p*, pore (sensillum placodeum); *sb*, sensilla basiconica; *Mp*, maxillary palpus; *St*, stipes; I, II, III, segments one, two, and three of the maxillary palpus.

Henig (1931) has mapped the nervous system in the antennæ of *O. lota.* This can be taken as representative of a typical nerve map since it coincides with the nervous arrangement in the species which I have studied.

The maxillæ are located near the sides of the labium and fused to it at their bases. I follow here the nomenclature of parts employed by Henig (1931). Each maxilla has a large and fleshy base, the stipes (*cf.* Fig. 3). Distal to this is the palpiger. The remaining part of the appendage is the maxillary palpus. The first segment supports a conspicuous protuberance, the maxillary headpiece. Segment II is the same size as the headpiece, and Segment III is a minute barrel-shaped structure. Only the headpiece and the three segments contain any sense organs other than the usual types of tactile hairs. Following is a generalized outline of the sense organs occurring on these locations:

TABLE IV

Types of sensilla occurring on the antennæ and maxillæ.

Antennæ	Maxillæ
Sensilla basiconica	Sensilla basiconica
Large	Small
Small	
Sensillum styloconicum	Sensillum styloconicum
Small	Large
Sensilla placodea	Sensilla placodea

1. Headpiece—three true hairs (sensilla trichodea), one to three very small cones (sensilla basiconica), two large sensilla styloconica.

2. Segment III—one true hair (sensillum trichodeum), one sensillum placodeum on the side opposite the location of the headpiece.

3. Segment II—one sensillum placodeum.

4. Segment I—one sensillum placodeum, five to seven minute cones (sensilla basiconica) on the extreme end of the segment.

This outline may serve as a description of any one of the species examined since the arrangement and structure of the sense organs is very similar. The descriptions of Henig for *O. lota* and of Grandi for *B. mori* differ but slightly.

The type of innervation of the maxillæ is similar to that of the antennæ and need not be described here.

From this survey we see that there are certainly many sense organs to which an olfactory function might be attributed. Table IV summarizes the types found.

It seems improbable that sense pores (sensilla placodea) are olfactory in function, although thus far only one direct experiment has been made to test this point. The maxillæ and the terminal segment of the antennæ of fifteen *E. kuehniella* larvæ were carefully removed by cutting. This operation left the larvæ with sense pores on the stump of the antennæ in addition to many located on the thoracic legs. The larvæ completed a normal life cycle and not once during this time did they respond to an odor.

Brues (1920) suggested that the odor of a plant may be one factor influencing food plant choice by lepidopterous larvæ. It is now possible to test this suggestion by direct experiment and, moreover, to check the results by ascertaining whether they are consistent with the various characteristics of the chemoreceptors of caterpillars as they have been described in the foregoing sections.

I chose to work, for the most part, with the larvæ of *Anosia plexippus*. The first series of experiments deal with the rôle of olfaction on plant choice.

Both sides of the leaves of a clean vigorous milkweed plant (*Asclepias syriaca* L.) were liberally painted with a cheap perfume having a characteristically penetrating odor. As soon as the perfume on both surfaces of the leaves had become dry the leaves were stripped from the plant and placed on the floor of a clean, odorless, wooden breeding box. The leaves still retained the pungent odor of the perfume. A larva of *Anosia plexippus* was then introduced into the cage. Under no circumstances would it eat the leaf. It crawled about and acted toward the leaf as it would towards any foreign leaf. The same experiment was repeated using methyl alcohol. At first the larva did not approach the leaf, but after a short time approached and began to nibble the leaf and eventually ate it. The leaf at first gave off the odor characteristic of methyl alcohol. This odor is very pronounced to our own senses; therefore, on the basis of threshold experiments for olfaction it must have been perceptible to the larva.[1] When the larva finally nibbled the leaf, I found that no odor remained and that there was no detectable alcohol taste on the leaf. Since man's threshold is lower than that of the larva, it would seem that the larva likewise was not able to detect any alcohol taste, and, therefore, ate the leaf.

It is common experience that when larvæ are placed in a breeding cage, they come to rest on the leaves of their food plant. The following experiments are based upon this observation.

An assortment of various leaves was cut into uniform squares and placed in a breeding cage. Eighty-five per cent of the total number of

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[1] Experiments indicate that the relationship between the olfactory thresholds of man and caterpillars remains the same for practically all odorous substances. Dimethyl phthalate, which is odorless to man, appears to be odorless to caterpillars. Larvæ show absolutely no sign of being able to perceive this substance.

Monarch larvæ came to rest on the squares of milkweed only. If, however, the squares of milkweed were treated with perfume or turpentine, the larvæ were found resting indiscriminately on any square. If every square was treated with either of the two substances mentioned, the larvæ again were found resting on any square, without so much as nibbling a piece of the leaf. Here it appears that the perfume and turpentine obscured that property of the milkweed by which the larvæ recognized it. The following experiments designated as "screen tests" prove definitely that this property is odor.

Whatever leaves were used in the tests were laid on the floor of the breeding cage. A square of clean wire screen whose area coincided with that of the cage floor was pressed over the leaves and fastened tightly into position. Care was taken that no portion of the leaves projected through the screen. A larva was then placed in the cage. As it crawled about its path was plotted and various movements of its head noted. The diagrams represent typical paths (*cf.* Fig. 4).

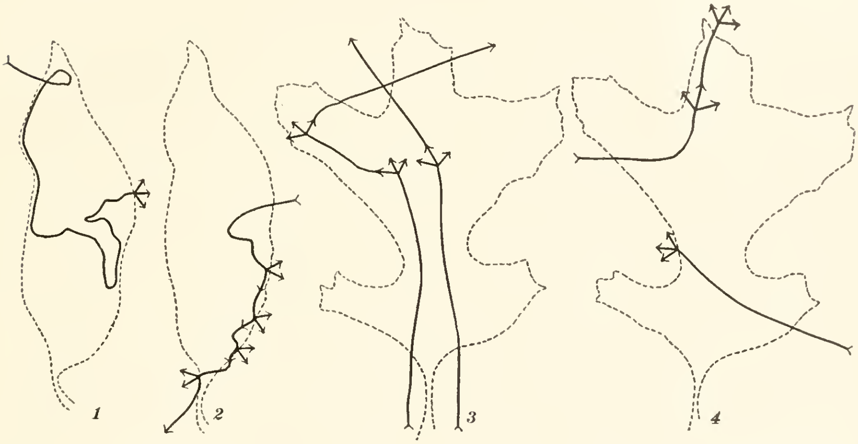

FIG. 4. 1 and 2. Diagrams of typical paths of *A. plexippus* larvæ over their food plant (milkweed) during a "screen test." 3 and 4. Diagrams of typical paths of *A. plexippus* larvæ over a foreign leaf (oak) during a "screen test." The grouped arrows indicate locations where the larvæ felt around in all directions.

Monarch larvæ passing over "foreign" leaves maintained a straight path. When they passed over a milkweed leaf, they described a zigzag path, a sort of "feeling about." They never, with one exception, tried to bite through the screen or to get at the leaves in any other manner. When they tended to pass off the leaf, however, they raised the anterior portions of their bodies and felt about. In 50 per cent of the cases the larvæ turned back over the leaf. The paths for various species varied only slightly.

The most significant experiment in this series consisted in placing the following leaves under the screen: mullein (*Verbascum Thapsus* L.), oak (*Quercus ilicifolia*), plantain (*Plantago major* L.), mullein coated with milkweed latex, oak coated with milkweed latex, and plantain coated with milkweed latex. The paths of the larvæ showed definitely that the caterpillars recognized the latter three leaves immediately.

If, as these experiments seem to indicate, larvæ recognize the milkweed by some odor that the leaves emit, leaves coated with some odorless but disagreeable (to the larvæ) tasting solution should, nevertheless, attract the larvæ. All of the foregoing experiments were repeated. Leaves were covered with either sucrose solutions, sodium chloride solutions, or hydrochloric acid solutions.

Although larvæ immediately recognized milkweed leaves coated with a sodium chloride solution, they would begin to eat such leaves but never continue eating them. The leaf had the correct odor but the wrong taste. It is significant to note that although larvæ would not eat salt-covered leaves, they, nevertheless, came to rest on them in preference to foreign leaves.

In conclusion, two more types of tests were tried. The first consisted of painting various substances such as oak leaves, cherry leaves, lettuce leaves, and filter paper with milkweed latex. The second consisted of stripping of both surfaces of a milkweed leaf and gluing them on either side of some other kind of leaf. The sticky milkweed latex was used as a glue.

In the first case the larvæ would attack the treated substance and reject it after a few mouthfuls. At intervals they would attack it afresh but would starve rather than eat it. In the second case the same was true. The normal behavior of the larvæ was to attack the edge of the leaf. When they did this, however, they soon tasted the foreign leaf sandwiched in between the milkweed. A few larvæ managed to survive by eating both surfaces and leaving the middle leaf untouched.

We may conclude that the odor of the leaf and its taste are primary factors influencing food plant choice in lepidopterous larvæ.

DISCUSSION

Experiments have been presented which show definitely that olfaction and gustation are the primary factors influencing food plant choice by lepidopterous larvæ. The threshold, location, and probable end organs of each of these two senses have been determined.

Smell in caterpillars is a sense of exceedingly "short range" since, in general, the threshold for food plant odors is very high. A larva, therefore, cannot scent its food plant from afar. The caterpillar, if it was not hatched on the food plant, must find its food entirely by chance. It is a common occurrence in nature for larvæ to be knocked or blown from the plant upon which they were feeding. Gravity and light direct them to where their food plant would normally be. For example, larvæ which feed on a ground plant such as plaintain or clover are positively geotropic and negatively phototropic, while larvæ which feed on trees, shrubs, or herbaceous plants which grow upright are negatively geotropic and positively phototropic. Excellent proof that the first move in the direction of the food plant is a chance movement can be found in the case of the larvae of *Echœtias egle* which feed on milkweed. After vigorous wind storms the brightly colored larvæ are found on practically every type of upright vegetation in the vicinity of a milkweed patch. Those which are not lucky enough to chance upon a milkweed plant most certainly die unless after another tumble to earth they ascend the proper plant. Similar conditions throughout nature must account for a large part of the mortality of caterpillars.

Having found a plant, the caterpillar crawls over the leaf, waving its head from side to side. The larva's "short range" sense of smell is particularly fitted to this environment since the larva moves about with its olfactory receptors, the antennæ and maxillæ, exceedingly close to the source of stimulus, the leaf. We recall that the olfactory threshold of caterpillars appears to be no better than our own. We also find by pressing various leaves to our noses that many of these leaves which we previously thought of as possessing no odor, do emit a rather characteristic one. The caterpillar, then, with its poor threshold perceives many plant odors because their sources are very close at hand. Plants which appear odorless to us are probably odorless to caterpillars. The world of the Monarch larva is probably composed solely of "milkweed odor" and "not milkweed odor." It is possible that the odor of milkweed is the stimulus which sets off a reflex action, namely a biting reflex. At this point the larva "tastes" the leaf. Probably the taste substance in the milkweed is a stimulus which causes additional reflexes culminating in feeding.

When we attempt to explain the feeding habits of polyphagous larvæ, we encounter various difficulties. The experiments presented proved that the senses of gustation and olfaction are just as acute in this type of caterpillars as in monophagous and oligophagous species. There are, however, many plants whose odors are undoubtedly below the thresholds of the larvæ. Then again in these larvæ a specific odor may not set off a biting reflex as postulated for monophagous species. Any plant, therefore, which does not have repelling odor or whose odor is below threshold is attacked. Likewise a plant which does not have a repelling taste substance or whose taste substance is below threshold is attacked. If there is a similarity of odor and taste in several plants, as for example, cabbage and radish, it is conceivable that a fundamentally monophagous caterpillar is deceived into feeding on both species. I am inclined to classify as oligophagous only those insects which feed on plants with similar odors and tastes. An example would be *Pieris rapæ* whose larvæ feed on cabbage and radish, both of which taste and smell alike to humans. All other species are polyphagous. They eat any plant which does not contain a repellent. A polyphagous larva is not one which recognizes many odors, but rather a larva in which a particular taste and odor is not required to start the chain of feeding reflexes.

When we attempt to find what constituent of the plant leaf attracts the larva, we must remember certain basic facts: The constituent must most probably be one which will stimulate the human senses of taste and smell because experiments seem to indicate that the thresholds of caterpillars are higher than those of humans; many of the constituents of a leaf vary with the season, the age of the leaf, and the time of the day and the attractant must be a constituent of the leaf which does not vary under the above conditions.

The latter statement needs explanation. To human beings cabbage always tastes and smells like cabbage, lettuce like lettuce and so forth, regardless of the time of day or season the vegetable is grown or eaten. Obviously the substance in such a vegetable which gives it its characteristic taste and odor must remain relatively constant. It may vary slightly in concentration but must never fall below threshold concentration. Since it appears that caterpillars can neither taste nor smell better than we can, the substance in the food plant by which the larva recognizes it undoubtedly remains quite constant in concentration. Thus in examining a plant for this unknown, we may reject all substances which are peculiar to particular times and conditions.

Using this method we may hope to determine what substances attract different larvæ to different plants. In the case of the milkweed butterfly experiments seem to indicate that the substance determining the choice of this species of larva is the milkweed latex or some substance contained therein. The compound or compounds in the latex which give it its characteristic taste and odor are the attractants which we seek.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA

I. COLOR CHANGES AND DIURNAL RHYTHM IN LIGIA BAUDINIANA

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In the summer of 1936, while engaged in collecting near the biological station at St. George's West, I noticed distinct color differences in specimens of an isopod that was very common on the rocky ledges along the shore. The animals on the black porous rock above the high-tide mark were dark-grey or black, while those feeding on the algæ and other plant material covering the limestone of the intertidal zone were always yellowish-white in color. Closer observation led to the belief that this difference in coloration was due, not so much to morphological variations in pigment distribution, as to an active physiological concentration and dispersion of pigment within cells. It was therefore decided to investigate more fully the chromatophoral behavior in these isopods which were identified as *Ligia baudiniana*.

The mechanism of chromatic change in the isopod crustaceans has not been definitely established. Following the early studies of Pouchet (1876) on the color changes of decapod crustaceans, Matzdorff (1883) published a report on the coloration of *Idotea*. He was of the opinion that chromatophoral activity was under the control of the nervous system, but decided that the inconclusive results obtained by severing the ventral nerve cord were to be attributed to injury brought about by the operative manipulation. Menke (1911), in a study of the rhythmic activity of color changes in isopods, believed that the mechanism for such responses was based upon physiologically innervated melanophores, referring in support of this to a figure of an innervated chromatophore from the integument of a young *Philoscia* in Weber's (1881) paper. It appears from a study of the figure that the nerve fiber supplying the chromatophore is a process of a peripheral nerve cell, the other processes of which supply the sensory (?) hairs on the body. The relationship, however, is not very evident and no further anatomical detail is given. The implication drawn from the illustration

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[1] This study was made possible by a grant from the James F. Porter Fund of Harvard University. I wish to thank Dr. J. F. G. Wheeler, Director, for the many kindnesses shown me during my stay at the Bermuda Station.

was that chromatophoral activity depended upon a local receptor-effector mechanism, a condition which subsequent study of blinded animals failed to confirm (Tait, 1910). Menke reported that section of the ventral nerve cord had no effect on metachrosis, but that the chromatophores became dispersed if the dorsal side of a segment were cut without damaging the dorsal vessel.

Several years later, Perkins (1928) and Koller (1928) showed that pigmentary changes in the decapod crustaceans were under the control of an endocrine substance that had its origin in the eye-stalks. When appropriate extracts of the eye-stalks of *Palæmonetes* were injected into shrimps which had become dark as a consequence of their having been kept upon a black background, the animals soon became light due to a concentration of pigment within the integumentary chromatophores. Perkins' studies on *Palæmonetes* were confirmed and extended by Brown (1935), who believes that the chromatic components of the pigmentary system are controlled by separate hormones.

Extensive experiments by Kropp and Perkins (1933) and by Hanström (1935) established the presence of a humoral chromatophore activator in the eye-stalks of a wide variety of crustaceans. Hanström, in addition, has located two organs suspected of glandular function in the eye-stalks of many decapods, and one of these, the blood gland (*Blütdruse*), he believes chiefly responsible for the control of pigmentary activity. In some crustaceans, *Gebia affinis* and *Hippa talpoida,* these glands are present, not within the eye-stalk, but in the head on the surface of the brain. This was confirmed by experiments which showed that extracts of the eye-stalks of these two crustaceans when injected into blinded *Palæmonetes,* had no effect upon the dispersed chromatophores, while extracts of the heads were as active in effecting concentration of the pigment cells as were preparations from the eye-stalks of *Palæmonetes.*

In view of these advances in the study of color changes of decapod crustaceans, it was thought advisable to study melanophore activity in the isopods with regard to endocrine control.

Materials and Methods

Specimens were collected along the shore where the isopods are found in large numbers, feeding upon the plant material that is uncovered by the ebbing tide. The animals were obtained by lifting rocks and dropping them into a wooden bucket, the impact being sufficient to jar the individuals loose from the under sides of the stones. Collections were made daily to insure a supply of normal, active individuals.

In following the responses to changes in color of background, a large white porcelain dish, the bottom of which was covered with moist white sand, served as a white background. For adaptation to black backgrounds a glass bowl, the outside of which had been covered with black paint, was used, the sand for the bottom of this vessel being mixed with an equal amount of pulverized coal. The containers were illuminated by light from a 60-watt lamp at a distance of 18 inches.

It became necessary during the course of these experiments to observe the reactions of blinded *Ligia*. Blinding by extirpation of the sessile eyes was unsatisfactory because the ensuing hemorrhage invariably caused the death of operated individuals. Such operations were eventually abandoned and blinding was accomplished by covering the eyes with an opaque mass, obtained by mixing plaster of Paris and lampblack with a little water. This mixture was applied over the head so that the eyes were completely covered, and, after being allowed to dry, was coated with a thin layer of waterproof paste to prevent moistening and crumbling.

Extracts of the heads of *Ligia* were prepared in various concentrations to determine the possibility of an endocrine factor in pigmentary changes. These were prepared in two ways: in one, the heads were crushed and ground with 1.0 cc. of sea-water in a mortar, most of the coarse detritus was separated off, and the remaining fluid drawn directly into a hypodermic syringe for injection; the second method was essentially the same, except that the triturated heads were transferred to a test tube and brought to a boil. The heat was sufficient to clump most of the solid material so that the supernatant fluid was almost water-clear. The solution was allowed to cool and was then drawn into the syringe.

In the experiments where such extracts were injected, the pigmentary condition of the specimen was first examined by means of a dissecting microscope, and observations were again made within 10 minutes after treatment. Care was taken, when injecting, to insert the needle dorsally into the body spaces, well anterior to the heart (usually between the fifth and sixth thoracic segments), to avoid loss of body fluid. Control injections consisted of both boiled and ordinary sea water.

COLOR PATTERN

The dominant and most obvious component of the chromotophore system in *Ligia* consists of cells containing a black pigment, possibly a melanin. These pigment cells are distributed over the entire surface of the animal, being more numerous and apparently smaller in size on the dorsal side, especially in the region of the mid-line; they are less densely aggregated near the lateral margins of the tergites (Fig. 2).

A second component of this system consists of white pigment. This occurs in many individuals as rather large clusters on the posterior, dorsal surface, and, by examination with the low powers of the dissecting microscope, does not appear cellular. In addition to massed pigment there are, however, definite cells containing this white substance. Such "guanophores" appear to show a limited activity in the concentration and dispersion of their pigment, but close observation of their behavior was not undertaken in this study. A yellow pigment of some sort is also present and is most noticeable in preserved specimens. This combination of body colors is very effective in maintaining a concealing coloration of the animals in their native habitat.

TABLE I

Responses of the melanophores of Ligia baudiniana to changes in background.

Series I

Time after transfer of 7 dark specimens to a white background	Condition of melanophores			
	0	A	B	C
5 minutes		7		
10 minutes		1	6	
20 minutes			2	4
35 minutes			1	5

Series II

Time after transfer of above white specimens to a black background	0	A	B	C
17 minutes		1	5	
95 minutes	2		4	
240 minutes	4	1	1	
300 minutes	6			

Series III

Time after transfer of 7 dark specimens to a white background	0	A	B	C
5 minutes		7		
30 minutes			4	3

TABLE II

Responses of Ligia to injection of extracts. The concentration per cc. designates the number of heads triturated in 1.0 cc. of sea-water. E_w, extract prepared from white-adapted specimens. E_b, extract prepared from black-adapted *Ligia;* A_b, black-adapted isopods were injected; A_w, white-adapted animals were injected. In Series I, the extracts were prepared from background-adapted animals, in Series II extracts were similarly prepared and boiled. Extracts for Series III were prepared unboiled, from specimens during the two conditions of diurnal activity, while in Series IV similar extracts were boiled previous to injection.

Concentration per cc.	E_w into A_b					E_b into A_b					E_b into A_w				
	No. of Ligia inject.	0	A	B	C	No. of Ligia inject.	0	A	B	C	No. of Ligia inject.	0	A	B	C
Series I															
4	11	2	3	6											
4	10	2*	3	5											
4	9		4	5											
4	10	1	1	8											
8	10	1	4	5											
4						14	1		13		3				3
Total	50	4	15	29		14	1		13		3				3
Series II															
25	3			3											
8	5		1	3	1										
4	10			2	8										
10						4			4		4				4
Total	18		1	8	9	4			4		4				4
Series III															
4	11			11											
4	10			10											
4						8	7	1							
4						8	3	5							
4						14	1	5	8						
4						5	1		4						
Total	21			21		35	12	11	12						

TABLE II (cont.)

Series IV

Concentration per cc.	E_w into A_b					E_b into A_b					E_b into A_w				
	No. of Ligia inject.	0	A	B	C	No. of Ligia inject.	0	A	B	C	No. of Ligia inject.	0	A	B	C
10	4				4										
5	4			4											
2.5	4			1	3										
1.25	4			1	3										
10						4			1	3					
5						4			1	3					
2.5						6	2	3	1*						
10						4				4					
5						4		1	3						
2.5						4		2	2						
1.25						4			4						
4						4	1*	1*		2	4				4
2						4		1	2	1					
1						4	1*		1	2					
0.5						4		2	2						
0.25						4		4							
0.12						4		3	1						
0.06						5	1	3	1						
4						8	2*		2	4					
Total	16			6	10	67	3	19	20	19	4				4

* Specimen died.

COLOR ADAPTATIONS TO BACKGROUNDS AND EFFECTS OF BLINDING

The surmise that there was a physiological color change in adaptation to the color of the background was confirmed by testing the responses of specimens in the laboratory on black and on white backgrounds (Table I, and Figs. 2 and 3). The melanophore changes of the black-adapted *Ligia* of Series I and Series III were recorded after the animals were transferred to a white background. The specimens of Series II were those of Series I which had become adapted to the white background and were then transferred to the black vessel as a converse experiment. The conditions of the melanophores recorded in Tables I and II are designated by the symbols used in Fig. 1. The color changes

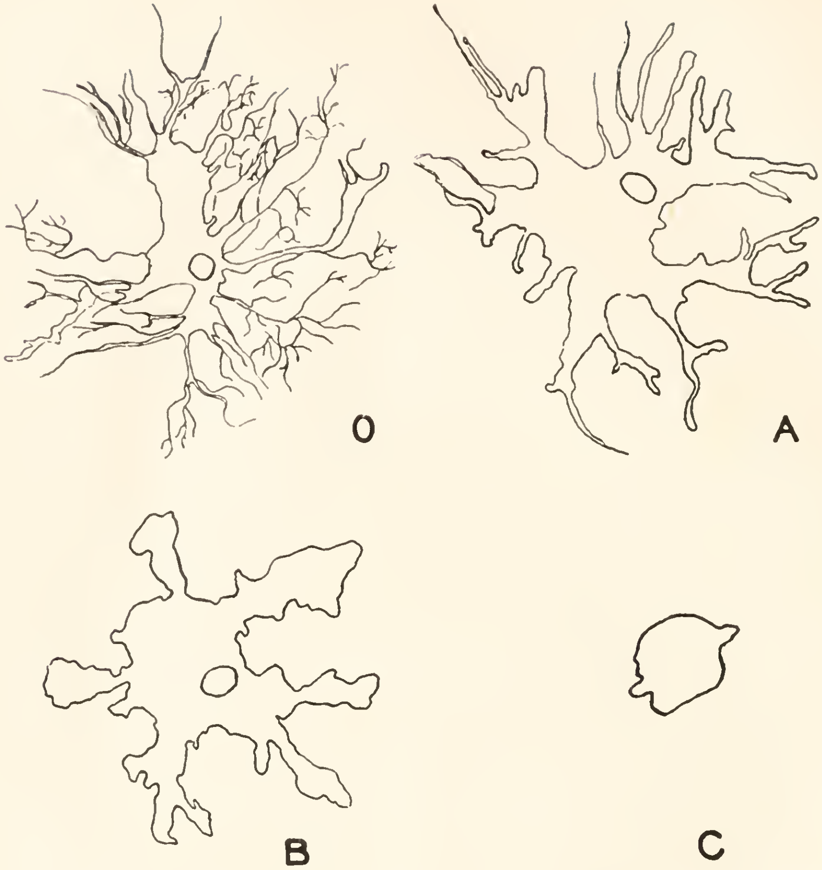

FIG. 1. Outline drawings of melanophores, showing the conditions of the cells in four stages from dispersion to concentration of pigment. *O*, the maximally dispersed state, with many delicate processes visible; *A*, beginning of concentration, the distal processes of the cells losing their delicate tracery, and ending in blunt rounded knobs; *B*, the stellate condition; *C*, the punctate state, with the nucleus obscured by the pigment granules.

in response to background are seen (Table I) to be more rapid during concentration than during dispersion, being in this respect similar to the behavior of the chromatophores in *Palæmonetes*.

It became desirable after these preliminary experiments to observe the color reactions of blinded *Ligia*. For the isopod crustaceans, Tait (1910) reported darkening of *Ligia oceanica* when the eyes were covered with an opaque mass, and Piéron (1914) obtained similar results with blinded *Idotea*. In the decapods, however, differing types of responses have been found, depending upon the method of blinding employed by the investigator, and upon the crustacean studied. In operating upon the pedunculate eyes of the decapods, two methods of blinding are possible. One procedure is to remove or destroy the retina only; the second method is to excise the entire eye-stalk, thereby removing Hanström's blood-gland in addition to the retina. The pigmentary changes in the animal resulting from the second type of operation are due more to deficiency of the chromatophorotropic hormones from the blood stream than to destruction of the retina.

With *Palæmonetes,* which does not possess a melanophore system in the body pigment, Brown found that cauterization of the retina resulted in a loose concentration of the red pigment and a half-way dispersed condition of the yellow pigment cells. Ablation of both eye-stalks, however, effected a full dispersion of the red and the yellow chromatophores and random variations in the white pigment cells. Carlson (1935, 1936) in studying *Uca pugilator,* which possesses melanophores in the pigmentary system of its integument, found that removal of the distal thirds of both eye-stalks had no effect on the pigment cells, whereas total removal of both stalks (the blood-gland is located in the middle third of the eye-stalk) caused a permanent concentration of the melanophores and the erythrophores, while " the yellow chromatophores were slightly more contracted than the stellate state, and the white ones a little more expanded than that state." Abramowitz (1935) also reported concentration of the black pigment cells and dispersion of the guanophores of *Portunus anceps,* following total removal of the eye-stalks. The pigmentary reactions of the two brachyurans to these operations parallel the color changes of many of the lower vertebrates after hypophysectomy. There is a striking similarity in physiological effect between crustacean eye-stalk extract and the melanophore-dispersing principle of the vertebrate hypophysis, as reported in recent studies by Abramowitz (1936*a,* 1936*b*).

In preliminary tests a number of *Ligia* were blinded by removal of the eyes with a spear-point needle. Such specimens, with their melanophores initially punctate, became darker after the operation, but it was thought advisable to repeat the experiment, using a technique that involved less injury to the animal. Similar results were obtained when 10 white-adapted *Ligia* were blinded by covering the eyes with an opaque mass. The melanophores of all 10 isopods became maximally dispersed within an hour; five minutes after this last observation (3:45 P.M.) the specimens were placed in the dark-room, and when examined later in the evening (5:30 P.M.) 4 of them were light, 3 were intermediate, and 3 were still dark. The next morning (at 8:00 A.M.) of 5 surviving animals, 4 were dark and 1 was intermediate in color.

These confusing results became more intelligible when further observations showed that the isopods underwent a diurnal rhythm in melanophore activity in constant darkness. Under such conditions the pigment in the black cells was dispersed during the day and concentrated at night. Upon an illuminated black background, however, the rhythm did not appear at night; the isopods remained dark. The following notes on a series of animals in the dark-room indicate the pigmentary condition of the isopods:

June 23 10:30 P.M.	Six white *Ligia* with melanophores punctate were placed in the dark-room.
June 24 9:00 A.M.	Two specimens are still light; 4 are dark with the melanophores dispersed.
June 24 6:30 P.M.	Same as at 9:00 A.M.
June 24 10:45 P.M.	Six specimens are light: in 3 the melanophores are punctate, while in the remaining 3 they are punctate and stellate.
June 25 10:15 A.M.	All specimens are dark: 4 with melanophores maximally dispersed; 2 show them stellate and slightly more dispersed.
June 26 12:30 A.M.	All 6 isopods are light with the melanophores punctate.
June 26 10:30 A.M.	Six specimens are dark.
June 26 4:30 P.M.	Same as at 10:30 A.M.
June 26 10:15 P.M.	All the animals are light.

Unfortunately the critical times during the day when these rhythmic changes were initiated could not be determined. It became evident that considerable variation existed in the onset of the changes, and that such variation might be due to the effects of captivity. There could, however, be no doubt of the existence of a pigmentary rhythm, since it was observed repeatedly both under laboratory conditions and at night in the natural habitat of the isopods.

Effects of Injecting Extracts

Hanström (1935) showed that the activity of crustacean eye-stalk extracts in concentrating the dispersed chromatophores of blinded *Palæmonetes* was correlated with the presence of the blood-gland in the

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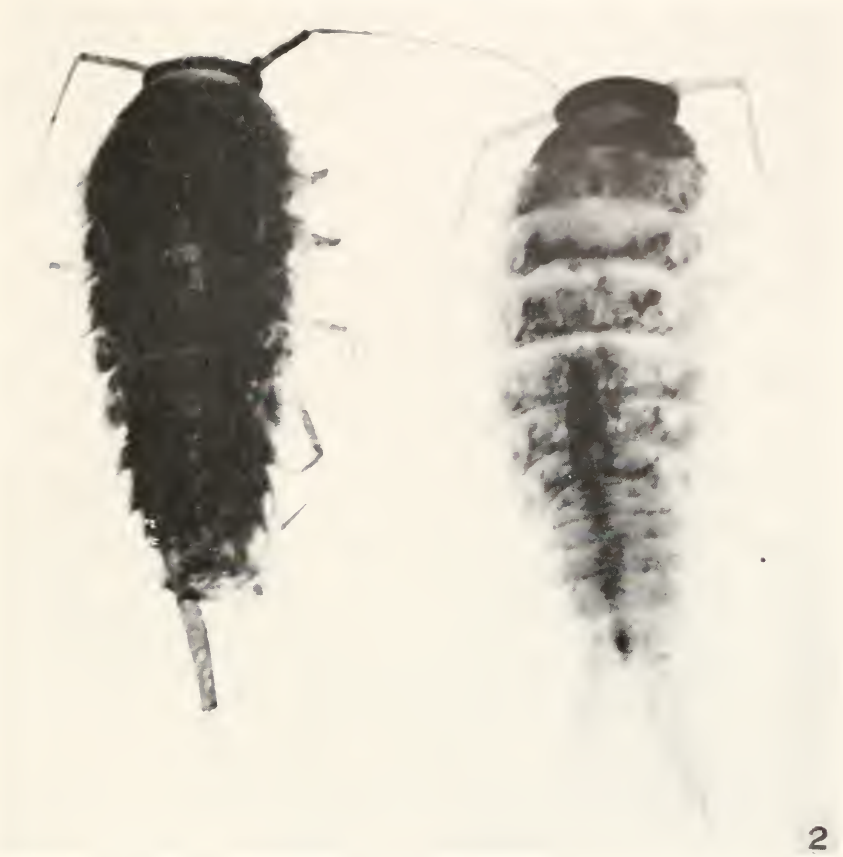
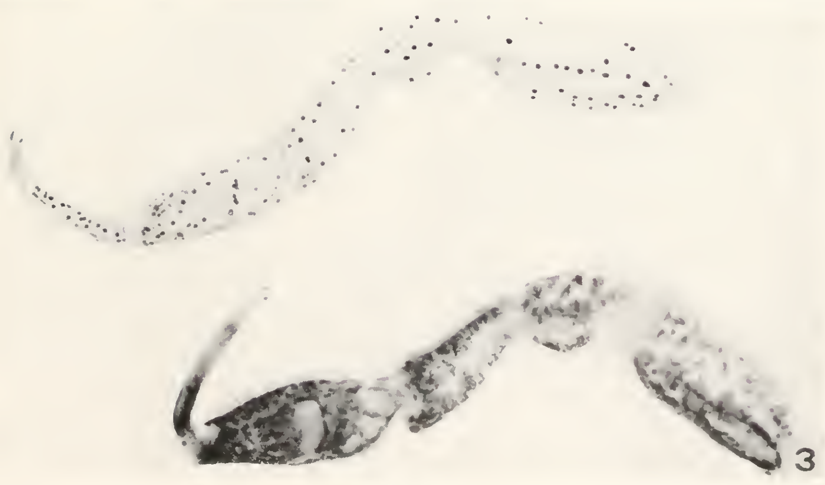
EXPLANATION OF PLATE I

FIG. 2. On the left is a specimen that has been darkened by exposure to a black background; on the right an isopod adapted to a white background. The photograph was taken from *Ligia* which had been killed with hot water and preserved in formalin.

FIG. 3. Appendages from white-adapted and black-adapted individuals, showing the two extreme conditions of the melanophores.



2



3

PLATE I

eye-stalks used. Stalk extracts from the eyes of *Gebia affinis* and *Hippa talpoida* were ineffective in concentrating dispersed chromatophores because the blood-gland is absent from the eye-stalks of these crustaceans. Extracts prepared from the heads of these two decapods are active because the organs presumed to be of endocrine function are located on the surface of the brain. Since the eyes of *Ligia* are sessile, entire heads of specimens were used in preparing extracts. The bodily changes in color following the injection of extracts prepared from specimens in different pigmentary conditions are indicated in Table II.

It is evident that black-adapted specimens responded to such treatment by a concentration of their melanophores, while white-adapted isopods showed no change. Control injections of sea-water into 42 black-adapted *Ligia* were without effect on the dispersed melanophores of 37 individuals, while 5 animals became perceptibly lighter in color. These 5 specimens were part of a group of 14 isopods that were injected at night (11:15–11:45 P.M.), a time when specimens in the dark-room are light because of the diurnal periodicity.

The behavior of the black pigment cells, following injection of the extract into *Ligia*, is in striking contrast to that of the melanophores of *Uca*. Carlson (1936) and Abramowitz (1936*b*) have shown that the dark coloration may be restored to blinded *Uca* by the injection of eye-stalk extracts. The responses of the melanophores of the two brachyurans when compared with the diametrically opposite behavior of those in *Ligia* may be due to some fundamental difference in the nature of the pigment cells (*vide*, Bigney, 1919, on the responses of retinal and body pigments of frogs to adrenalin), or it may be due to the existence of two different hormones, one causing concentration and the other effecting dispersion of the black pigment. Unfortunately, critical experiments to decide this second possibility were not performed.

The phenomenon of a diurnal rhythm in the activity of the chromatophores and retinal pigments of crustaceans is now well known through the work of Keeble and Gamble, Menke, Piéron, and Welsh. The basis for this activity is, however, less well understood. Piéron believed the color changes of *Idotea* to be due to a nervous mechanism, and said of the rhythmic changes, " the nervous centers can periodically control the reflex without being directly stimulated by the sensory impressions (received by the eyes)." In view of recent developments showing that the pigmentary activities of the body and the retina are under hormonal influence (Perkins, 1928; Kleinholz, 1936), Piéron's explanation should be revised to allow for the humoral factor.

Several interesting speculations as to the basis for this diurnal activity have been put forward by Welsh (1936) and by myself. Welsh suggests, "There may be a rhythmic secretory cycle in the gland which continues under constant conditions or the situation may be much more complex and the rhythm in the eye may only accompany a general rhythmic activity which results from a series of changes involving the nervous-endocrine systems." A third possibility, supplementary to the first suggestion, is that the rhythm may be due to a diurnal cycle of exhaustion and elaboration of the secretory material when the animal is maintained under constant conditions.

Physiological tests fail to substantiate this last possibility. Examination of the data in Table II shows that extracts prepared from specimens of *Ligia* in the two diurnal pigmentary conditions are practically equally effective in causing concentration of the dispersed pigment in the melanophores. The greater activity of boiled extracts has been reported in similar observations by Perkins and Snook (1931) and by Hanström (1935).

There is as yet no direct evidence favoring either of the two remaining possibilities. More complicated reactions than are at present indicated may be involved in such periodic pigmentary changes. While neither of these hypotheses really clarifies the means by which the rhythmic activity originates, such assumptions are of assistance in narrowing down the number of systems to be studied in the hope that eventually more light may be thrown upon the nature of this phenomenon.

Summary

1. The bodily changes in color of *Ligia baudiniana* upon black and upon white backgrounds are due chiefly to a dispersion and concentration of pigment granules within melanophores.

2. When the animals are kept in constant darkness, there is a diurnal rhythm in pigmentary activity, the isopods being dark during the day, and light at night.

3. Injection of aqueous extracts of heads into the body spaces of dark *Ligia* brings about lightening in color by a concentration of the melanophores.

4. Extracts from the heads of dark and of light specimens in the two conditions of diurnal rhythm are practically equally effective in concentrating the melanophores of dark isopods. It may be concluded from this that the diurnal pigmentary activity is not due to a cycle of exhaustion and elaboration of secretory material in the endocrine gland controlling the color changes.

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THE MITOTIC RATE IN TADPOLE SKIN AFTER REPEATED INJURY[1]

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The epidermis of frog tadpoles usually shows a very low rate of mitotic division (Cameron, 1936*a*, 1936*b*). The present report is based on an attempt to set up a situation highly favorable to active mitosis. It was thought important to ascertain whether the consistently low rates obtained had been determined by specific inhibiting factors related to the age and development of the animal, the time of year, the existence of mitotic rhythms, or the manner of feeding and maintaining the tadpoles used in previous work.

MATERIAL AND METHODS

Bullfrog tadpoles about six centimeters long were used. Care was taken to reject those showing signs of metamorphosis. Each was kept in a one-liter beaker half full of water, the beakers being surrounded by a water bath at 25° C. Eight 150-watt Mazda lamps, inside frosted, with reflectors, were grouped 18 inches above the water level in the beakers. Constant illumination was provided from 6:00 A.M. to 9:00 P.M. daily. Black curtains excluded stray light during the remaining hours. The bath and lights, with their electrical control system, were kindly supplied by Professor Albert Saeger.

After the tadpoles had been 24 hours in the bath, the posterior half-centimeter of the tail of each was cut off and fixed in Bouins fluid. Successive half-centimeter pieces were taken at 24-hour intervals. Each cut was approximately at right angles to the tail axis. Beginning with the second amputation each piece of tissue had for its anterior face a freshly cut surface and for its posterior face a surface which had been cut 24 hours earlier. The four lateral faces were covered with original epidermis which had contributed cells for the covering, by migration, of one or more " posterior face " surfaces.

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[1] This study was aided by a grant from the Committee on Radiation of the National Research Council to W. C. Curtis.

[2] Research Fellow in Biology, Harvard University. Fellow of the General Education Board.

Each block of tissue was cut into vertical sagittal sections 10 μ thick and stained with Mayer's hæmalum and orange "G." Thus each microscopic section of any piece, after the first or tail-tip piece, had along its posterior border a surface over which cells had migrated during the previous 24 hours, unless the section was a lateral surface section.

TABLE I

Data from a tadpole in which each injury was covered by migration within twenty-four hours.

Days	Previous injuries	Average cells per section	Sections counted	Mitoses counted	Mitoses per 10,000 cells
1	0	12,800	35	51	1.1
2	1	2,100	26	6	1.1
3	2	2,340	30	9	1.3
4	3	4,000	36	18	1.3
5	4	1,800	49	8	0.9
6	5	2,400	45	11	1.0
7	6	950	60	5	0.8
8	7	2,300	64	12	0.8

TABLE II

Data from a tadpole in which all but the last two injuries were covered by migration within twenty-four hours.

Days	Previous injuries	Average cells per section	Sections counted	Mitoses counted	Mitoses per 10,000 cells
1	0	10,800	14	56	3.7
2	1	10,040	11	35	3.2
3	2	4,800	12	18	3.1
4	3	2,150	13	5	1.8
5	4	3,800	21	15	1.9
6	5	5,100	22	22	2.0
7	6	3,800	31	151	13.2
8	7	3,500	42	732	50.0

The mitotic figures in all the epidermis of every fifth section of each piece were counted at a magnification of 660 ×. Mitotic figures were found in the original epidermis and very rarely in "new" epidermis formed by migration within 24 hours after injury. Rates per 10,000 cells have therefore been taken from the ratios of total mitoses to number of non-migrated epidermal cells. Average numbers of cells per section were obtained by counting selected sections from the region midway between the first lateral and the center sections of each piece of tissue.

Findings

Tables I and II are samples of the results obtained, one for each of the two classes into which the tadpoles studied can be logically separated.

DISCUSSION

The experimental animals, living under conditions favoring a high rate of general metabolism, were subjected to successive injuries each requiring a greater number of cells to cover it on account of the increasing diameter of the tail cephalad from the tip. The epidermal cells anterior to any injury were subjected to some degree of stimulation to migrate over the cut surface and to divide, assuming that injury is the source of a stimulus to mitosis. The increase, if any, in relative frequency of mitosis in regions anterior to and near the successive wounds might be taken as an index of the degree to which mitosis supplied the new cells required.

In the first animals studied no increase in the mitotic rate was found. In cases where seven injuries were followed by seven complete coverages (see Table I), the rate remained around 1 in 10,000. Table I is a sample of counts made by K. O. Mills. The report (Cameron and Mills, 1936), made at the General Meeting of the Marine Biological Laboratory for 1936, was based on these data. The rate here is definitely lower than the rate found in adult frog skin adjacent to areas injured by X-rays (Cameron, 1936*b*, Table I[3]).

There were some individuals in which the latest and most extensive injuries were not completely covered by "new" epidermis after 24 hours. Counts of these specimens show that the mitotic rate rises sharply in the neighborhood of areas not covered in the usual manner. Table II is an example of records of this class. The injured areas of the second through the sixth pieces were covered, the seventh had an uncovered area about one-third the diameter of the notochord, and the eighth had an uncovered area about the diameter of the notochord.

The mitotic rate in the seventh is about five times the average rate of the previous six, and the rate of the eighth about twenty times the same average. The conditions associated with incomplete coverage within 24 hours may then be credited with a twenty-fold increase in the mitotic rate, and it is inferred that the low rates in the other cases indicate that cells were being supplied through migration. The same general picture is found in other specimens.

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[3] This table is for 10,000 cells, not for 1,000. The figure 1,000 in the title is a misprint.

It is also possible that there is simply a cumulative or additive effect of all the injuries to a given tadpole which sets off a period of division as soon as the required threshold is attained. Certainly the conditions leading to failure of rapid coverage of the injured surface are closely related to those producing an increased mitotic rate. It also seems clear that even skin which has maintained a very low rate for a long period can be stimulated to active proliferation.

SUMMARY

Successive half-centimeter pieces were cut each day for eight days from the tails of bullfrog tadpoles. The epidermis maintained a very low rate of epidermal mitosis despite the great loss of cells by migration over the injured surfaces. Conspicuous exceptions with relatively high rates were found in cases where the epidermis failed to cover the seventh or eighth wound within twenty-four hours after injury. Here the mitotic rate reached a value twenty times the previous average rate.

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OBSERVATIONS ON TWO TYPES OF RESPIRATION IN ONCHIDIUM[1]

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Onchidium is a small, naked, pulmonate mollusk which is remarkable because of several conspicuous peculiarities. The habitat of *Onchidium* is littoral-marine and the animal divides its existence between life in the water and in the air. A daily sally from the cavernous nests in the eroded rock in which these animals live is followed by a return governed by precise homing behavior (Arey and Crozier, 1921). The mantle of some species bears eyes with retinulae of the inverted type—a condition unique among gastropods (Semper, 1877; Stantschinsky, 1908; Hirasaka, 1912). The presence of lungs, except as analogues adapted from a portion of the kidney, was long denied, but this error has since been corrected.

Cuvier (1805) was the first to describe lung cavities in *Onchidium,* but this observation gave way later to other interpretations. Milne-Edwards (1857) considered the pouch hitherto described as lung to be kidney, like that of other gastropods. This view was revived by v. Ihering (1877), who interpreted the so-called lung of *Onchidium* as being comparable to the broadened cloacal portion of the kidney of some other marine forms. This he thought had undergone a partial functional change from a primarily secreting organ to a respiratory sac. The conclusions of v. Ihering were enthusiastically supported by Joyeux-Laffuie (1882). On the other hand, Semper (1876) argued for the existence of two separate though juxtaposed organs and in this opinion he was joined by Bergh (1895) and especially by v. Wissel (1898). As the results of the investigations of these latter workers, the *Onchidium* family became removed from the nudibranchs and now rests securely once more among the pulmonates.

As to the mode of respiration employed, Cuvier considered it to be solely pulmonary. In opposition stand Ehrenberg (1831), Milne-Edwards (1857), v. Ihering (1877), Vaillant (1871), Joyeux-Laffuie (1882) and others who have argued for two types of respiration, cutaneous and pulmonary, but with the latter playing a subordinate rôle.

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[1] Contribution No. 241. The observations were made at the Bermuda Biological Station for Research, Inc.

Only v. Wissel has considered the lungs to be the major organs of respiration when the animal is out of water.

Recently an opportunity has come to supplement earlier studies (1919*a*; 1919*b*; 1921) on the natural history and behavior of *Onchidium floridanum* Dall, an Antillean species common at Bermuda. Among the new observations are some dealing with respiration. Since the opinions expressed hitherto have not always been drawn from the living animal, the recording of certain direct observations should be of value and interest.

Onchidium floridanum spends the major part of its existence in eroded cavities in the soft Eolian limestone which constitutes the rocky shores of Bermuda. These cavernous nests, often the size of one's fist or larger, are intertidal in position and communicate to the outside by small, cleft-like apertures so overgrown with *Modiolus* as to be wholly inconspicuous. It is remarkable to observe how *Onchidia*, some 2 cm. in length, can insinuate themselves through such narrow openings. Once a day, but only during daylight hours, the animals of a colony leave their nest to wander about on the neighboring intertidal rocks, feeding on the felt-work of alga which carpets them. Emergence is at a fixed time when the receding tide has dropped a definite distance below the nest. The feeding period lasts for an hour or so, after which a return is made to the appropriate home nest through the operation of precise homing activities, as has been related elsewhere (Arey and Crozier, 1921). The emergence and return of a colony involves a roughly simultaneous group activity, although it seems clear that not all members of a communal group come out at every feeding period. Certain it is that on some occasions relatively few individuals are to be seen in the open, while during stormy weather or when the proper tidal period comes too close to darkness all animals may stay within their places of refuge. During the periods of several hours, twice a day, when the nests are left above tidal level, the *Onchidia* are for the most part exposed to the air, although retained water and water seepage from the porous rock keep the nest very wet. By contrast, during the feeding period the animals are commonly exposed to the direct rays and baking heat of the semitropical sun.

The mantle of *Onchidium floridanum*, like most of its allied species, is thickly studded with large numbers of tiny, rounded elevations which can properly be designated as short papillæ. These are far smaller than the long, branching ones figured by Plate (1894) for *O. savignyi* and *O. verruculatum*. Moreover, the peculiar tegmental eyes with inverted retinulæ, as described for some species by Semper (1877) and others, are lacking. The vascular relations in these short papillæ can be inferred from the injections of Joyeux-Laffuie, who has described the arrangement of blood vessels in similar papillæ of *O. celticum* as constituting a rich network supplied by an afferent and an efferent vessel. The sum total of this vascular, tegmental plexus furnishes an adequate structural basis for cutaneous respiration. On the other hand, the paired pulmonary sacs are cavernous structures which are also well vascularized. They are situated near the caudal end of the animal and open by a prominent, sphincter-guarded aperture located in the midplane just behind the foot.

It would seem that the respiration carried on under water must be of a cutaneous, tegmental nature. This conclusion is enforced by the observation that the pulmonary aperture closes and remains tightly shut when the animal is submerged. The adequacy of prolonged cutaneous respiration for the needs of the animal is shown by the fact that specimens can be kept submerged for a month without any noticeable impairment. Nevertheless, there is one further characteristic feature of the aquatic life of *Onchidium* which merits mention with respect to cutaneous respiration. When submerged, these animals tend to be highly inactive and this necessarily reduces their oxygen requirements to a low level. Yet it is difficult to devise experiments which will reduce mantle respiratory exchanges to a degree that clearly proves the indispensibility of this organ in respiration. Attempts to eliminate mantle respiration through the administration of coats of collodion, varnish or even stiff vaseline lead to technical difficulties, and in some instances unphysiologic involvements, so that crucial evidence fails.

The proof that the lungs are actually used, but only while the *Onchidia* are active and exposed to the air, is sufficiently convincing. In the water the closing of the pulmonary vent is a reflex through water contact, as is shown by the following experiment. If, while the aperture is open in the air, the animal be flooded locally with water, the sphincter closes and remains closed as long as it is surrounded by water. In the air, on the other hand, the pulmonary aperture not only opens but becomes a prominent orifice, 0.5 mm. or more in diameter. When the animal then crawls about, the posterior portion of its mantle characteristically elevates; sometimes this is on one side, but usually the extreme posterior end is raised into an arch, high above the substrate, leaving the pulmonary aperture fully exposed. If an animal be held on its back and vaseline be then smeared over this opening, the orifice opens from time to time and a bubble of air is forced into or through the vaseline. Similarly, when an *Onchidium* is returned from air back to water, a small air bubble usually appears soon at the pulmonary vent. In the air such an unbroken bubble can at times be observed to disappear by being drawn into the lung cavities, only to reappear again. Attempts to render the lung sacs functionless through injecting their cavities with vaseline were not successful due to the closed nature of the system.

In the absence of quantitative measurements it is impossible to conclude with any finality as to which mode of respiration in air is the more important. Most writers have considered cutaneous respiration as primary and have relegated the lung sacs to a subordinate position. On the other hand, v. Wissel alone credits the lungs with performing the chief respiratory work under these conditions since he believes that slime, secreted in the air to counteract drying, is an effective block to tegmental gaseous interchanges. This conclusion probably does not rest on a secure basis since the degree to which slime would render the mantle functionless in air is quite problematical. To carbon dioxide it should constitute no serious impediment although oxygen probably diffuses less readily. Nevertheless, the slime coat is never thick and, moreover, it is my experience that although *Onchidia* emerging from a nest are apt to bear a slime pellicle, yet this is usually scraped off by the *Modiolus* that crowds the entrance. Certainly animals feeding in the open are generally rather clean of such investments. But even though the lungs may take over the major rôle of respiration in the active animal out of water, one must not overlook the fact that *Onchidia* may live for long periods in the air under conditions where pulmonary respiration is seemingly excluded. Thus, animals placed in a covered glass jar, partly filled with water, tend to frequent the air rather than the water and for the most part become highly inactive. When assuming such inactivity they contract down against the glass in a hemispherical mass; by contrast, the shape of the crawling animal is definitely elongate, with a length some three times the body breadth. When quiescent and contracted, with mantle and foot in close contact with the substrate, it seems extremely doubtful that pulmonary respiration can be brought into play. In this semidormant state the animals may remain entirely inactive for long periods, perhaps for a day or more. It is possible that this observation signifies that cutaneous respiration in air is more efficient than in water. It may well be that interchanges with the air take place through the moist, papillate mantle better than with water which is relatively low in oxygen content. In any event, most individuals select the air-filled half of the jar.

The higher efficiency of respiration in air is perhaps supported by a further observation. Animals whose mantles had been subjected repeatedly to faradic stimulation in order to exhaust their repugnatorial glands were returned into a jar of water. Such stimulation constitutes a sufficiently severe treatment so that some animals may succumb. Most of this particular lot remained quiescent on the bottom of the jar and by the next day were in poor condition. On the third day they were immobile and scarcely responsive to tactile stimulation; some had already died. They were then transferred to a moist, but not water-filled, jar, whereupon most of them improved remarkably and some time later seemed recovered. Other animals subjected to similar faradic treatment, but placed in a moist jar from the beginning, kept in good condition.

Conclusions

Onchidium floridanum respires cutaneously, and chiefly through its numerous though short mantle papillæ, both when submerged and when quiescent in the air. Under both of these environmental conditions the pulmonary aperture remains closed and the lungs are inoperative. When given a choice, most animals select the air for long periods of quiescence.

When an animal, out of water, is actively crawling, the posterior portion of the mantle is raised from the substrate to expose the opened pulmonary orifice and facilitate pulmonary respiration, the existence of which is demonstrable. The lungs could be of considerable service during such periods of activity and it may be that they actually dominate the terrestrial respiration of active animals. Such a conclusion, however, does not rest on any sound evidential basis and the lungs may serve merely as accessory respiratory organs when the animals are out of water and active.

If the lungs be subordinate in the terrestrial respiration of active *Onchidia,* in addition to their disuse during aquatic life and inactive air existence, then the lungs of this pulmonate (which has secondarily acquired marine habits) have indeed sunken to a low functional state.

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OVARY TRANSPLANTS IN DROSOPHILA MELANOGASTER: STUDIES OF THE CHARACTERS SINGED, FUSED, AND FEMALE-STERILE

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INTRODUCTION

In *Drosophila melanogaster* several mutant types are known which are characterized by female sterility. Three of these, singed (*sn*, 1–21.0; bristles and hairs have singed appearance), fused (*fu*, 1–59.5; longitudinal veins 3 and 4 fused at base), and female-sterile (*fes*. 2–6 ±; ovaries undeveloped), have been studied by transplanting ovaries from mutant flies to wild type hosts and vice versa in order to determine whether, under these conditions, there is any detectable influence of the tissues of the host on the development of the implant

In connection with the transplants involving singed, an attempt was made to determine the influence of certain variations in genetic constitution and of relative development on the frequency with which an implanted ovary becomes attached to the genital ducts of the host. These studies of frequency of attachment were made primarily for the purpose of providing a measure of the usefulness of the method of gonad transplantation in *Drosophila* as an experimental tool.

MATERIALS AND METHODS

The methods of collecting eggs, culturing larvæ, and of making the transplantation operations were essentially similar to those given by Ephrussi and Beadle (1936). Unless stated otherwise, all transplantation operations were made on individuals in the late larval stage of development within 24 hours of puparium formation, and donor and recipient larvæ were of approximately the same absolute age.

Stocks of the three mutant types were made up in such a way that an eye color gene, known to affect pigmentation of the Malpighian tubes (Beadle, unpublished), was carried in the same chromosome as that in which the sterility gene in question is located. In this way larvæ homozygous for the sterility gene could be selected at the time the operations were made. The actual stocks used were $w^e sn/ClB \times w^e sn$, $w\ fu/ClB \times w\ fu$, and $fes\ lt/Cy, sp^2 \times fes\ lt$.

In those experiments in which it was desired to determine the nature of the eggs laid by a given female, mature females into which ovaries had been implanted were placed, singly, in vials, given fresh food, mated to males of the appropriate genotype, and observed for three or four days in order to note the kind of eggs laid and to collect any larvæ that hatched from them. Larvæ from individual females were transferred to culture bottles for determination of phenotype. At the end of three or four days each female was dissected and a record taken of the number of ovaries and the way in which they were attached to the oviducts.

In the experiments dealing with frequencies of attachment of implanted ovaries, advantage was taken of the fact that singed ovaries can be distinguished from wild type ovaries by the characteristic shape of mature or nearly mature eggs (shorter and blunter at the anterior end than are eggs produced in a wild type ovary). Thus, by aging mature females for two or three days, the eggs in the ovaries develop sufficiently so that, by dissection, it is a simple matter to determine whether or not the implanted ovary is attached to the oviduct of the host.

TRANSPLANTATIONS INVOLVING SINGED

Females homozygous for the gene singed are known to produce eggs visibly different in shape from those produced by wild type females; such eggs never hatch (Mohr, 1922). Singed males show normal fertility, and oöcytes of the constitution *sn*/+ give rise to reduced eggs, *sn* in constitution, which develop normally after fertilization with either sn^+ or *sn* sperm. It is clear, then, that the two types of eggs, *sn* produced by a *sn* female and *sn* produced by a *sn*/+ female, are differentiated by the constitution of the mother. This differentiation might be brought about directly in the developing oöcyte or indirectly by means of an influence of other maternal tissues. The experiments reported below were carried out in an attempt to differentiate between these two possibilities.

Ovaries from $w^e sn$ larvæ were transplanted to F_1 wild type larvæ derived from the cross of the two inbred wild stocks, Florida and Swedish-c. From 37 larvæ into which had been injected $w^e sn$ ovaries, 27 gave rise to adult females. These were mated individually to $w^e sn$ males. Two females were lost before any record of the eggs was made, and one before dissection. Of the 24 females on which complete records were secured, it was found that 13 laid two types of eggs, one type judged by appearance to be *sn*, the other wild type. The remaining 11 females laid only wild type eggs. When these females were dissected, it was found that the attachment of the ovaries to the ducts agreed in every case with the egg-laying record (Table I). Of the 11 females which laid only wild type eggs, 5 had no developed implanted ovary. Only eggs of normal wild type appearance hatched and all hatched eggs that were tested by growing the larvæ to maturity gave rise to wild type flies. It is clear that none of these normal eggs originated in the implanted *sn* ovary.

Table I

Summary of the results of transplanting singed ovaries to wild type female hosts and the reciprocal. In this and following table wild type is designated by +.

Implant	Host	Number of mature females	Eggs laid	Total progeny		Result of dissection, implant		
				Phenotype	Number	Attached	Free	Not determined
w^esn	+	13	+ and *sn*	+	746	13*	0	
w^esn	+	6	+	+	624	1†	5	
+	w^esn	6	+ and *sn*	+	321	4	0	2
+	w^esn	4	+	+	26	2	0	2
+	w^esn	3	*sn*	—	0	0	2	1

* In one female three ovaries were attached to ducts, in a second, only one wild type ovary present; see footnote to Table II.

† Three ovaries attached to ducts; see footnote to Table II.

In making the reciprocal transplants, wild type ovaries transplanted to w^esn larvæ, 34 operations were made. Seventeen adult females were obtained which were mated to w^esn males. A record of the eggs laid was secured on 13, and the attachment of the ovaries on 8. The w^esn females were less vigorous than wild type females and were inclined to stick to the food mass or on the moist walls of the vials. As indicated in Table I, the egg-laying record and the dissection records agree in all cases. Here again only the eggs which were judged to be wild type gave rise to offspring and these were all wild type; they must have originated in wild type implanted ovaries. This result shows that, aside from the production of abnormal eggs, the reproductive apparatus of a singed female is able to function in an essentially normal way, i.e., the genital ducts, accessory glands, and external genitalia of such a female are functionally normal.

It is evident from the results just described that there was no detected influence of host on implant or of implant on host, i.e., the implants developed according to their own genetic constitutions with no apparent influence of the genetic constitution of the host tissues. So far as the experiments go, the differentiation is intragonadal in nature, but the possibility of extra-gonadal influences, acting before the time at which the transplants were made, is not excluded.

TRANSPLANTATION OF FUSED OVARIES

Fused females lay eggs of normal appearance but which fail to hatch when fertilized by sperm from fused males. According to Lynch (1919) such eggs show signs of development and in very exceptional cases (2 in several hundred) may give rise to larvæ which die at an early stage of development. Lynch (l. c.) observed, however, that if such eggs are fertilized by X sperm from a wild type male (or other not-fused males) normal development occurs. Absence of males from such matings indicates that fertilization by a Y sperm does not bring about normal development. Eggs from *fu*/+ females, even though they be *fu* in constitution after reduction, show normal development when fertilized with normal X, fused X, or Y sperm. Fused males are apparently normal in fertility. As pointed out by Lynch, a *fu* egg can be made good by something which happens to it before fertilization (development from a +/*fu* oöcyte) or, if it develops from a *fu*/*fu* oöcyte, by being fertilized by a not-fused X sperm. Assuming that the effect of the fu^+ gene is a positive one, we might express this in terms of gene activity in the following way. A *fu* egg arising from a *fu*/*fu* oöcyte lacks something essential for its normal development. If a *fu* egg arises from a *fu*/+ oöcyte, this deficiency has been made good before fertilization by the activity of the fu^+ gene directly in the oöcyte (possibly after fertilization by the activity of the fu^+ gene in the polar body nuclei), or indirectly by the activity of the fu^+ gene in cells other than the oöcyte. Whatever is deficient in a *fu* egg arising from a *fu*/*fu* oöcyte can evidently be compensated for by the activity of the fu^+ gene brought in by an X sperm. The results of transplanting a *fu* ovary to a not-fused female should answer the question of whether the fu^+ gene can influence the oöcytes indirectly through tissues outside the ovary.

White fused (*w fu*) ovaries were implanted in $w^e sn$ hosts. Singed females were used as hosts so as to be able to distinguish the eggs of an implanted ovary from those of the ovaries of the host. Of 37 $w^e sn$ females in which fused ovaries had been implanted, 18 laid both singed and fused eggs, 3 fused eggs only, 14 singed eggs only, and in 5 cases no eggs at all were laid. Females laying fused eggs and mated to fused males gave no offspring. After three days, 6 females that had been laying fused eggs were isolated and remated to wild type males. From the eggs laid during the next three days some larvæ hatched.

These were collected and placed in a culture bottle en masse. In all, 28 flies emerged from larvæ collected in this manner; all were wild type females.

In this experiment, as in the case of the analogous experiment with singed ovaries, the implanted fused ovaries behaved in all respects in the same way as would have been expected had they completed development in their original environment. Conclusions similar to those arrived at in the experiments with singed are indicated.

Experiments with Female-Sterile

Females homozygous for the gene *fes* have rudimentary ovaries; they can be distinguished from normal females by dissection one day or more after eclosion. Apparently the oöcytes fail to grow normally. Homozygous *fes* males are fertile.

Transplantation of wild type ovaries to *fes* females show that such an ovary can become attached to an oviduct of the host and function normally. Of 7 such females in which the implanted ovary had developed, 2 produced wild type eggs which, when fertilized with sperm from *fes* males gave rise to wild type adult flies. Five females showed the implanted ovary normally developed but unattached.

Of 4 wild type females in which implanted female-sterile ovaries had been implanted, 2 had the implant attached and in 2 the implant was unattached. In both cases, after aging of the females, the implanted ovaries showed no more development than is characteristic for the ovaries of normal *fes* females. It is clear from the two instances in which an implanted *fes* ovary replaced a normal wild type ovary of the host that *fes* ovaries are capable of competing successfully in attachment with normal ovaries.

The experiments with female sterile ovaries show that, under the conditions of the experiments and with respect to the characters under consideration, the development of ovary implants is autonomous or independent of the genetic constitution of the host.

In experiments in which it is desired to recover eggs from ovaries grown in hosts of a different genetic constitution, the character female-sterile promises to be of considerable value. When one uses *fes* females as hosts, no eggs develop in the ovaries of the host and all recovered eggs therefore originate in the implanted ovary. Furthermore, the limited development of *fes* ovaries minimizes the unfavorable effects of mechanical crowding often apparent in females with three normally developed ovaries. Females homozygous for the *fes* gene with an implanted normal ovary have been observed to lay an average of more than 25 eggs per day for an interval of 10 days with no evident signs of decreased production at that time.

FREQUENCY OF ATTACHMENT OF IMPLANTED OVARIES

As pointed out above it is possible, by using donors and recipients which carry a gene difference at the singed locus, to determine by dissection of aged females whether or not an implanted ovary has established connection with an oviduct of the host.

Experiments were carried out which have a bearing on three questions: (1) Is a singed ovary on a par with a wild type ovary in establishing a connection with an oviduct? (2) Do variations in the difference in genetic constitution between ovaries competing for attachment influence the result? (3) What is the effect, on competition for attachment, of a difference in development of competing ovaries (age difference between donor and recipient)? Three stocks were used:

1. F_1 $w^e sn$ females from the cross of two distantly related $w^e sn$ stocks, $w^e sn/ClB$ females mated to $w^e sn$ males from the stock $\widehat{yy} \times w^e sn$.
2. F_1 wild type females from the cross of Florida and Swedish-c inbred wild type stocks.
3. Inbred wild type stock Oregon-R-c, made homozygous an unknown number of generations previously by the standard inversion technique.

The results of two sets of reciprocal transplants involving these stocks are summarized in Table II. In the first pair of reciprocal transplants, involving the two outcrossed stocks, the percentages of attachment are 75.0 and 65.3. Neither of these is significantly different statistically from the 66.7 per cent expected on the basis of random attachment, and both are higher than the percentage (45.4) found by Ephrussi and Beadle (1935) in a series of miscellaneous experiments. Furthermore, the difference between the two values is not statistically significant. The second pair of reciprocal transplants, involving the outcrossed $w^e sn$ stock and the inbred Oregon-R-c wild type stock give percentages of attachment of implanted ovaries of 44.7 and 41.7. These two values, again approximately equal, are significantly lower than the corresponding values obtained in the first pair of reciprocals, and are significantly lower than the 66.7 per cent expected on the assumption that attachment is random.

It can be seen that in each pair of reciprocal transplants the frequency of attachment of wild type implants is higher than that of singed implants. However, in each instance the difference is of doubtful statistical significance. Combining the two series so as to compare wild type in $w^e sn$ with $w^e sn$ in wild type, values of 65.3 and 53.6 per cent are obtained, a difference aproximately 2.5 times its probable error. While it cannot be concluded that there is no inherent difference between singed and wild type ovaries with respect to the chance of their becoming attached to oviducts of the host, the experiments fail to demonstrate such a difference and show that, if it does exist, it must be of relatively little importance as compared with other differences.

From the data tabulated in Table II it is evident that the results are different with the outcrossed and inbred wild type stocks. Furthermore, reciprocal transplants give approximately equal frequencies of attachment of the implant, high in one case and low in the other. It is pointed out again that in these experiments the donors and recipients were approximately equal in absolute age (ages controlled to within a period of 2 hours or less).

Table II

Summary of data from experiments on frequency of attachment of implanted ovaries. In all instances listed at least two ovaries were attached to the two lateral oviducts of the host. In the calculated frequencies of attachment only the two classes with three ovaries developed, two attached, and one free, were taken into account. Probable errors are given with the calculated percentages.

Implant	Host	Three ovaries			Two ovaries *		Percentage attachment of implant
		Implant free	Implant attached	All attached *	Implant attached	No implant	
$_{+}$Fla/$_{+}$S-c	$w^e sn$	15	45	5	2	12	75.0±3.8
$w^e sn$	$_{+}$Fla/$_{+}$S-c	17	32	6	1	13	65.3±4.6
$_{+}$Ore-R-c	$w^e sn$	26	21	4	2	2	44.7±4.9
$w^e sn$	$_{+}$Ore-R-c	28	20	1	0	7	41.7±4.8

* In this and Table III, 26 instances are recorded of attachment of three ovaries. Two additional cases of such attachment are recorded in the footnotes to Table I. In many of the attachments of this nature one ovary appeared to be imperfectly attached. In no instance was a clear bifurcation of a lateral oviduct observed; often two ovaries appeared to have a common attachment to a single lateral oviduct. In several recorded instances only two ovaries were developed, sometimes both from the host (failure of the transplantation operation or possibly failure of the implanted ovary to develop), sometimes only one from the host (presumably injury to or destruction of a normal ovary during operation).

It seems reasonably safe to assume that the differences in frequency of implant-attachment observed between the two wild type stocks is to be attributed to differences in genetic constitution. The difference between the two series and the approximate equality in attachment frequency in reciprocal transplants suggest, and are consistent with, the following assumptions:

1. Which two of three ovaries will become attached is a matter of chance if the ovaries of the host and the implanted ovary are at the same developmental stage at the time of attachment.

2. If the implanted ovary is at a different developmental stage from the ovaries of the host (either more or less advanced) at the time of attachment, the implant will be at a disadvantage in competition with the ovaries of the host.

3. Development of ovaries takes place at different rates relative to absolute age in females of different genetic constitutions.

It should be possible to test directly the first two of these assumptions by varying the relative ages of donors and recipients. There is no way of determining except by trial whether the low value obtained in the series involving the Oregon-R-c wild type stock is the result of slower or of faster development of the ovaries of Oregon-R-c relative to those of the w^esn stock. If the former is the explanation, then by implanting ovaries from Oregon-R-c females into older w^esn females, the frequency should be decreased. If the latter is the case, then ovaries from Oregon-R-c females implanted into older w^esn females should show an increased frequency of attachment. Four combinations with a given age-difference are obviously possible with two given stocks.

Table III

Results of transplants involving w^esn and Oregon-R-c (Ore-R-c) with an age difference between donor and recipient.

Implant	Host	Implant younger or older	Age difference	Three ovaries			Two ovaries		Percentage attachment of implant
				Implant free	Implant attached	All attached	Implant attached	No implant	
			hours						
Ore-R-c...	w^esn	Younger	16–23	6	27	2	2	11	81.8±4.5
w^esn......	Ore-R-c	Older	16–24	0	1	1	0	0	——*
w^esn......	Ore-R-c	Younger	21–26	53	53	7	0	16	25.4±3.5

* Hosts died after pupation, see text.

Table III summarizes the attachment frequencies of implants in experiments made with the Oregon-R-c and w^esn stocks where the average age difference between donor and recipient was approximately 20 hours. It is seen that Oregon-R-c ovaries transplanted to older w^esn females give a significantly higher frequency of attachment than was obtained in the comparable experiment without an age difference (81.8 per cent as compared with 44.7 per cent). The reciprocal of this trans-plant, $w^e sn$ ovaries in younger Oregon-R-c females, should, following the assumptions made above, likewise give a high attachment frequency. However, when the experiment was made it was found that the hosts lived for more than 24 hours, appeared to pupate normally, but for the most part died before maturity. Four sets of transplants were made at separate times, and in a total of 149 operations, only two females reached maturity. As compared with this high mortality, the average mortality for all other experiments involving an age-difference was 32.2 per cent; the mortality for the transplantation operations involving no age-difference was 45.5 per cent. Apparently, for some reason, the implant was lethal to the host in this particular case.

In the only other combination attempted, $w^e sn$ ovaries implanted in older Oregon-R-c hosts, the frequency of attachment was lower than in the same combination without an age difference (25.4 as compared with 41.7 per cent, a difference of approximately 2.9 times its probable error).

The two successful combinations with an age difference are consistent with the assumptions listed above and indicate that, if these assumptions are correct, the ovaries of Oregon-R-c females are developmentally further advanced than are those of $w^e sn$ females of a corresponding absolute age. However, in the absence of the reciprocals of these age-difference experiments, the assumptions are by no means proved to be correct. The lethal result in the one combination is quite unexplained and suggests either that the assumptions are incorrect or that they fail to take into account all of the factors concerned.

Summary

The development of singed ovaries transplanted to wild type females in the late larval stage shows autonomous development. Eggs recovered from such females have the characteristic shape of eggs from singed females and they fail to give rise to larvæ. Wild type ovaries grown in singed hosts likewise show autonomous development. Viable eggs can be recovered from such ovaries; they give rise to wild type offspring (females heterozygous for $w^e sn$) when fertilized by $w^e sn$ sperm.

Fused ovaries grown in singed hosts have characteristics not detectably different from such ovaries grown in their normal position. Recovered eggs fertilized by *fu* or by Y sperm fail to hatch, but those fertilized by not-*fu* X-carrying sperm give rise to normal females heterozygous for *fu*.

Ovaries from female-sterile females grown in wild type hosts may become attached to the oviducts of the hosts, competing successfully with normal ovaries, but they remain rudimentary as they do in their normal genetic surrounding. Wild type ovaries grown in female-sterile females show autonomous development. Viable eggs giving normal development are recovered following attachment of the implant to the oviduct of the host.

Using a single outcrossed stock of $w^e sn$ and two wild type stocks, one outcrossed and one inbred, reciprocal ovary transplants show: (1) that the frequency of attachment of the implant varies with different genetic stocks, and (2) that, under the conditions of the experiments and with the numbers involved, there is no statistically significant difference in the frequency of implant-attachment in reciprocal transplants.

Transplants in which donors and recipients were different in absolute age show that the frequency of attachment of the implant can be varied, either increased (in certain combinations) or lowered, by varying the relative ages of donors and recipients. In one combination in which ovaries were implanted to hosts younger than the donors, there was apparently a lethal interaction such that most of the hosts died after pupation. The bearing of the age-difference experiments on the differences in frequency of implant-attachment observed with different stocks is considered.

The application of certain of the results summarized above to the use of gonad transplants in *Drosophila* as an experimental tool are pointed out.

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EYES OF DEEP SEA CRUSTACEANS

I. ACANTHEPHYRIDÆ

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The depths of the ocean impose upon the animals which live there a very different set of environmental conditions from those found at the surface. Absence of sunlight, low temperature, high pressure and viscosity, and a somewhat different chemical composition of the sea water necessitate morphological and physiological modification in the organisms which inhabit deep water. Some of these modifications are well known. For example, if we select the more obvious features which are associated with low light intensity, or even complete absence of sunlight, we must include the presence of photophores, peculiar pigmentation of the body, and adaptation of the eyes for vision at a very low intensity of illumination. The photophores and eyes of certain groups of deep-sea crustaceans and fishes have been studied in some detail in the past and are reasonably familiar to biologists. One of the first and best known studies in which the structure of the eye was related to depth was that of Beddard (1884) on the isopod genus *Serolis*. A similar study was made later (Beddard, 1890) on the genus *Arcturus*. Smith (1886) and Henderson (1888) had already noted the tendency toward degeneration in the eyes of deep-water crustaceans, but neither had made a detailed study of these eyes. Chun's (1896) study of the very unusual eyes of deep-water euphausiïds was made from collections of the " Challenger " as were Beddard's.

From the collections of the " Valdivia," Doflein (1904) obtained material for a valuable study of the eyes of deep-sea crabs. Dohrn (1908) described the eyes of a few other crustaceans taken by the " Valdivia." More recently Hanström (1932–33) has had an opportunity to study the eyes of deep-water crustaceans.

The problem which confronted these workers, and many others who have made more casual observations, was to explain why some forms found in deep water have well-developed and apparently functional eyes while others are completely blind or have very degenerate eyes. This still remains the most interesting problem in connection with a consideration of the effect of depth and hence of diminishing light on the eye. There are many more problems which the older investigators would have doubtless solved if they had had more material from known depths, living material, and a more constant supply.

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[1] Contribution No. 126.

The regular operation of the research vessel "Atlantis" of the Woods Hole Oceanographic Institution has made it possible to study further the animals of the deep sea and has led to the planning of a program in which the first step has been to investigate more fully the modifications with depth in the eyes of certain of the crustaceans. What happens to the size of the eye, the number of ommatidia, the amounts of reflecting and screening pigments, pigment migration, and the structure of the rhabdomes with increasing depth? What is the relation between the development of the eye and the presence or absence of photophores? Such questions, dealt with in an introductory manner, using for material certain representative deep-sea acanthephyrids or prawns, are those we wish to discuss in the present paper. Final conclusions must await the study of many more types of crustaceans from known depths, but certain tendencies are noticeable in the work thus far carried out.

The authors are indebted to several members of the Woods Hole Oceanographic Institution, particularly to Dr. H. B. Bigelow, and to Mr. B. B. Leavitt from whose collections, made in a study of the vertical distribution of deep-water plankton, some of the material for the present study was obtained. The expense of preparing the eyes histologically has been cared for in part by a grant from the Milton Fund of Harvard University.

METHODS

The first problem in understanding the effect of the physical environment on deep-sea animals is to obtain a series of a given species from known levels, sufficiently large so that it is possible to determine the average level at which such a species lives. The use of open nets is unsatisfactory as animals are caught at all levels, and only after a large number of hauls at a given station can an idea of the vertical distribution of a species be determined. Nets which may be sent down to the desired level closed, then opened and towed at that level, and finally closed before being brought to the surface are almost a necessity. To operate two to five such nets, particularly when towing at depths of one to two miles, presents mechanical problems which have been quite adequately solved. The closing net used in deep-water tows from "Atlantis" previous to 1936 is described in a paper by Leavitt (1935). The type of net used during the 1936 cruise was modified somewhat and was found to be well adapted for collecting material alive or in good condition. It will be described in a later paper.

The locations of stations from which material was obtained in 1933 and 1934 are given by Leavitt (1935). During July, 1935, two stations were made in the Sargasso Sea: Station No. 2462 (42° 29′ N. and 70° 21′ W.) and No. 2463 (42° 27′ N. and 70° 14′ W.). During late August, 1935, one station was made on the inshore side of the Gulf Stream: No. 2475 (38° 25′ N. and 71° 04′ W.). In September, 1936, collections were made at Station 2666 (39° N. and 70° W.) and at Station 2667 (35° 40′ N. and 69° 36′ W.).

Obtaining deep-water animals living and in good condition is a second difficulty. Where there may be a warm surface layer, as on the slope side of the Gulf Stream, the animals coming from deeper layers are subjected to a considerable increase in temperature which, it is believed, is much more damaging than the reduction in pressure. The following temperatures were found in early September, 1936.

Station 2666 Slope side of Gulf Stream		Station 2667 Sargasso Sea
Depth	Temp. °C.	Temp. °C.
Surface	21.0°	26.2°
200 m.	9.7°	18.3°
400 m.	5.4°	17.8°
800 m.	4.5°	14.9°
1000 m.	4.2°	11.7°
2000 m.	3.6°	4.3°

It may be seen that while there was a difference of 5.2° C. at the surface, at 400 m. there was a difference of 12.4° C. Many more of the larger crustaceans were living when they arrived at the surface at Station 2667 than at Station 2666.

During the two trips in 1935 an opportunity was had for the first time to study living forms. Among the acanthephyrids only one species, *Systellaspis debilis,* was obtained alive in any considerable numbers. The only requirement for keeping this form living was found to be a low temperature. At 10° C. they survived for three days, at the end of which time they were killed; therefore, at that time it was not known how much longer they would have lived. During the 1936 cruise a cooling system was on board which made it possible to maintain tanks of sea water at 5° C. Many more forms were taken alive and several species of crustaceans were kept living for the duration of the cruise. Apparently it is possible to maintain many deep-water forms alive if they are kept at a temperature relatively the same as that where they normally live.

The material used for histological study was fixed in Bouin's immediately on removal from the nets or, in cases where it was desired to observe pigment migration, was kept illuminated for a period of time before fixing. It was found quite necessary to cut through the carapace in order to obtain sufficiently rapid penetration of the fixative. The paraffin method was used in sectioning the eyes. In estimating relative amounts of pigment it was found desirable to leave some sections of each eye unstained. Ehrlich's hæmatoxylin and a counter of eosin were employed for staining those sections which were used in studying the general structure.

Material

Although certain species of the Acanthephyridæ are so numerous that the possibilities of fishing for these prawns on a commercial scale have been suggested, it was as late as 1881 before more than two species of the family were known. Today, due to the many deep-sea expeditions in the past fifty years, the family is represented by six genera and forty-five species. The acanthephyrids normally inhabit the deeper parts of the sea where the penetration of sunlight is practically immeasurable—the so-called " red prawn-black fish " region.

Like a number of other deep-sea animals which have been known to science for only a short time, the acanthephyrids are a comparatively primitive group. The biramous form of the legs, one of the most distinctive characters of the family, ranks them among the most primitive members of the decapod Crustacea. Their color, with but a few exceptions, is a uniform deep crimson red. In other respects the species may differ so noticeably from each other that a cursory examination would scarcely lead to their inclusion in a single family. The integument varies from a hard, polished, armor-like shell to a membranous skin which is displaced or torn from the slightest handling; the rostrum or " head-spine " is typically long and slender, but in many species it is almost entirely lacking; the legs may be short and comparatively stout, or long, slender and fragile, or they may be modified, as in *Ephyrina*, into broad, lamellate appendages; and the eggs are either so small that many hundreds may be attached to a single female, or so large that twenty-five would be a burden to even the larger species. It will soon be seen that this diversity of form is likewise illustrated by the eyes of these prawns. Since most of the species are strictly bathypelagic organisms, they probably obtain their food from the detritus that is constantly raining down from the swarms of minute plants and animals at the surface of the sea. They are peculiarly adapted to strain this food from their surroundings; the thoracic legs are all provided with numerous long hairs and spines which apparently form a very efficient, sieve-like basket when the legs are held curved beneath the body. In the genus *Ephyrina* the legs are strikingly wide and flat so that, when held in position, they perform their function in much the same way as the baleen of the whale-bone whales. This modification of the legs for food-gathering is of particular interest when one realizes that the thoracic limbs of most shrimp-like Crustacea are used primarily for walking on the sea-bottom and play no direct part in the swimming movements of the animal. Since many of the acanthephyrids probably spend their entire lives far above the sea-floor, the thoracic legs would be only a hindrance to the progress of the prawn through the water if they were not modified to perform a function entirely apart from that for which they were originally designed.

There are, of course, few barriers to the dispersion of bathypelagic organisms which inhabit a world-wide zone of comparative uniformity of temperature and salinity. Many of the Acanthephyridæ, as in most families containing a like number of forms, are known from a very few specimens and little can be concluded about the distribution of those forms at present; but of the commoner ones, some are practically cosmopolitan, some seem to have a discontinuous range, while still others are confined to a reasonably small area. The Indo-Malayan region has the largest representation of these species, if our present records are reliable, with the North Atlantic second in importance. It is a curious fact that there are at least three reasonably common species in the Indo-Malayan region which so far have not been encountered elsewhere, but all three of these species show a very close relationship to three other forms which have been found almost everywhere except in the Indian Ocean. Much more data must be accumulated before the factors responsible for the specific isolation of bathypelagic organisms are known.

As regards the vertical distribution of the group the available data are likewise incomplete. However, of the forty-five species, thirty have been taken with mid-water nets and must be bathypelagic for at least part of the time. Further investigation will undoubtedly reveal that at least some of the remaining fifteen species seldom go down to the sea-floor. On the other hand, three stout-legged, heavy-bodied forms which have always been taken with the dredge or trawl can safely be termed benthonic animals.

Investigations undertaken in the past few years by Mr. Leavitt on "Atlantis" have yielded invaluable information regarding the actual depths at which we may expect to find acanthephyrids in the North Atlantic. The accompanying tables (Tables I, II, and III) give the depths at which three of the most common Atlantic species have been taken with closing nets. Incomplete as these records are, it is quite evident that *Hymenodora glacialis* normally frequents a deeper zone than either of the other species, a fact which agrees with earlier data. *Acanthephyra purpurea* has been taken in open nets from the surface, where one specimen was collected with a dip-net down to 2800 meters, but the majority have been found between 400 and 1000 meters. *H. glacialis* has been found on two occasions at the surface in the Arctic, and some numbers of that species have been found in the stomachs of arctic sea-birds. The shallowest trustworthy record for the species taken with midwater nets, however, is one in which ten specimens were taken in 750 meters, and by far the largest number of specimens have come from

TABLE I

Depths at which specimens of Acanthephyra purpurea have been taken with closing nets by the "Atlantis."

Depth in meters	"Atlantis" Station	Number of specimens	Total specimens
300	1737	1	1
400	2263	10	10
800	2263 2462 2463	65 7 2	74
920	2216	10	10
1000	2260 2263	1 2	3
1400	2263	1	1
1600	2263	1	1
1800	2216	2	2
2200	2263	1	1
2600	2260	2	2

TABLE II

Depths at which specimens of Systellaspis debilis have been taken with closing nets by the "Atlantis."

Depth in meters	"Atlantis" Station	Number of specimens	Total specimens
400	2263	15	15
600	2260	1	1
800	2462	9	9
1800	2263	1	1

depths greater than 1000 meters. This species has also been taken with a closing net by the " Valdivia " Expedition in over 4200 meters in the South Pacific. *Systellaspis debilis* apparently frequents slightly shallower depths than the other two species, regardless of the fact that it is the only one of the three which has not been found at the surface. It has been collected with open nets between 32 and 2000 meters and appears to be most abundant between 150 and 500 meters.

At present little is known of the daily vertical migration of these

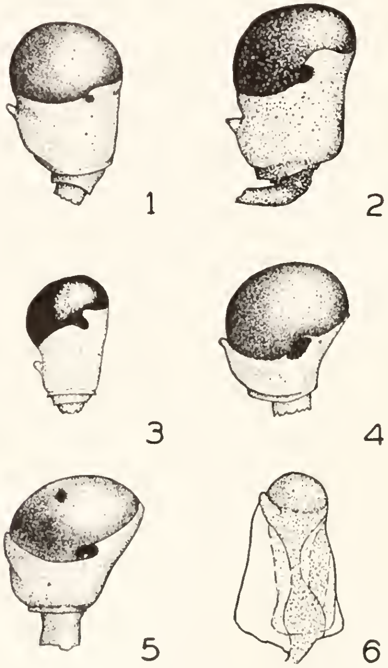
TABLE III

Depths at which specimens of Hymenodora glacialis have been taken with closing nets by the "Atlantis."

Depth in meters	"Atlantis" Station	Number of specimens	Total specimens
1000	2463	2	2
1200	2462	3	3
1400	2263	4	4
1600	2263 2475	2 3	5
2000	1737	3	3
2200	2263	1	1
2600	2260	2	2
2800 to 3200	1739	1	1

species. The results of past expeditions seem to indicate a very slight movement toward the surface at night in *A. purpurea* and *S. debilis* but absolutely none in *H. glacialis* (Murray and Hjort).

An examination of the external features of the eyes of these and other acanthephyrids offers a partial explanation of the above findings. The form of the eyes, although extremely diversified in the group as a whole, remains fairly constant within the separate genera. The eyes of the species of *Acanthephyra* (Fig. 1) are of normal size, generally well pigmented with a dark brown pigment and, as in the other genera, the dorsal surface of the eyestalk is provided with an incomplete, deeply pigmented ocellus or accessory cornea, whose function is unknown. In some species the ocellus is complete and entirely distinct from the



(Figures 1–6 are dorsal views of the right eye and eyestalk)

Fig. 1. *Acanthephyra purpurea*, × 5.
Fig. 2. *Ephyrina benedicti*, male, × 4.
Fig. 3. *Notostomus longirostris*, small female, × 5.
Fig. 4. *Systellaspis debilis*, × 7.5.
Fig. 5. *Oplophorus grimaldii*, male, × 7.5.
Fig. 6. *Hymenodora glacialis*, male, × 7.5.

true cornea, while in others it may be partially fused with the cornea or even entirely absent. The eyes of *Ephyrina* (Fig. 2) and *Notostomus* (Fig. 3) while similar in size and shape to those of *Acanthephyra* have a jet-black pigment which remains for many years even in alcohol. In *Systellaspis* (Fig. 4) the cornea is considerably larger in proportion to the stalk than in the foregoing genera. The extreme size is attained in the species of *Oplophorus* (Fig. 5) in which the eye may be actually broader than long, and the cornea is generaly set diagonally on the end of the stalk. It is worthy of note that these large-eyed species of *Systellaspis* and *Oplophorus* possibly all possess photophores. The other extreme is found in the eyes of the two species of *Hymenodora* (Fig. 6). In these forms the corneal portion is reduced to a size which is considerably smaller in diameter than the eyestalk. On recalling the records concerning the vertical distribution, it will be seen that those species which have the largest eyes apparently frequent the shallower layers of water, while those with vestigial eyes, like *Hymenodora,* may be termed truly abyssal forms.

The accompanying graph (Fig. 7), despite inaccuracies that can scarcely be eliminated in measuring tissues so subject to alteration after preservation, clearly indicates that the eyes increase in diameter in proportion to the length of the carapace. It is also quite obvious that the degenerate eyes of *Hymenodora* increase in size very little with the growth of the animal. Perhaps the most striking fact brought out by these measurements, however, is that the eyes of *Oplophorus grimaldii* and *Systellaspis debilis,* two forms bearing photophores, are larger in relation to body size than are the eyes of those forms lacking luminescent organs.

Comparative Structure of the Eyes

Fortunately the three most abundant species of acanthephyrids in the North Atlantic and therefore those whose vertical distribution is best known, represent three possible trends in the development of the eye. *Acanthephyra purpurea* is found in greatest numbers within the photic zone, and as it shows a diurnal migration this species must be influenced by the penetrating daylight, although of extremely low intensity. Even in the Sargasso Sea, where the water is very clear, the intensity of blue light at noonday may be reduced to 0.5 per cent of the light at the surface, at a depth of 180 meters (Clarke, 1933). The penetration of other components of daylight into Atlantic waters may be found in a paper by Oster and Clarke (1935). *A. purpurea* is found far below 180 meters during the day; hence it must be subjected to a very low intensity of illumination.

Systellaspis debilis has much the same vertical distribution as *A. purpurea*, but this form possesses numerous photophores, and, as has already been pointed out, there is an apparent correlation between the size of the eye and the presence or absence of photophores, for species of *Systellaspis* and *Oplophorus* have larger eyes in proportion to body size than the other genera of acanthephyrids which lack photophores.

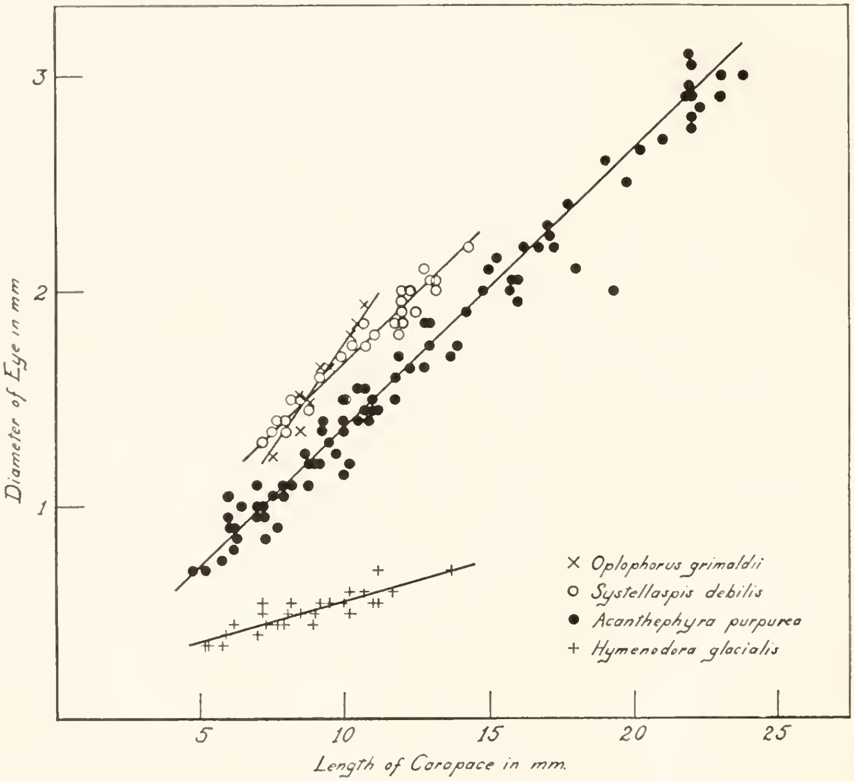


FIG. 7. The diameters of the eyes of four series of acanthephyrids are here shown plotted against carapace length. *Oplophorus* and *Systellaspis* have photophores.

The ability to emit clouds of luminous material is possessed by certain of the deep-sea prawns, as has been mentioned by Alcock (1902) and by Beebe, who observed this phenomenon frequently in his bathysphere descents. So little is known of this interesting method of illuminating the surroundings that it is not possible to hazard a guess as to its effect on the development of the eye.

The third species, *Hymenodora glacialis,* inhabits a region well below the photic zone and does not possess photophores and it will be shown that the eyes of this species are very degenerate structurally.

It will not be necessary to describe the general plan of the eye of acanthephyrids for it is basically like that of other decapod crustaceans, and such eyes have been described by Parker (1891), Patten (1887), Trojan (1913), to mention only a few of the earlier investigations of the histology of decapod eyes. From the standpoint of vision and visual acuity the most important features to be considered in any compound eye are the following: (*a*) number of ommatidia, (*b*) development of the rhabdomes (the receptor units), (*c*) amounts, distribution, and movements of screening and reflecting pigments. To these should be added the photosensitive material contained in the rhabdomes. So little is known of the nature of this material that it cannot be discussed at the present time. The average number of ommatidia in a longitudinal section of an eye may be taken as a measure of the total number of ommatidia and thereby the task of counting all the elements is avoided. In all counts the eyes of large, mature individuals were selected, although not the largest obtainable. In eyes of mature *A. purpurea* (Fig. 10) averaging 2.5 mm. in diameter the average number of ommatidia in a section was found to be 145. Eyes of mature *S. debilis* averaging 1.9 mm. in diameter had an average of 81 ommatidia. Eyes of *H. glacialis* averaging 0.6 mm. in diameter had an average of 22 ommatidia. The number of ommatidia, hence the number of rhabdomes, determines in part the visual acuity of an arthropod; therefore the variation in number in these three forms must be significant.

The rhabdomes, whence the fibers of the optic nerve arise, are in a sense the most important structures found in the compound eye. A single rhabdome is formed of parts of the seven functional retinular cells found in each ommatidium, and a longitudinal section presents a peculiar striated appearance. In none of the acanthephyrids which have been studied do the rhabdomes have the well-defined outlines seen in the majority of decapods, including certain deep-water forms. Of the three species being especially considered they are perhaps most definite in *S. debilis,* and may be seen in sections of the eyes of light-adapted specimens (Fig. 15) where they are outlined by the proximal pigment. In *A. purpurea* they are difficult to distinguish (Fig. 9). In all forms taken from the nets during either day or night they were never surrounded by pigment. This means that light entering a given ommatidium could reach the rhabdomes of neighboring ommatidia (Exner, 1891).

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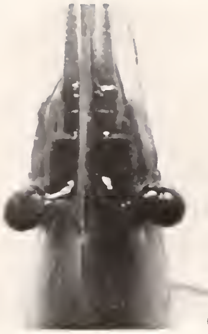
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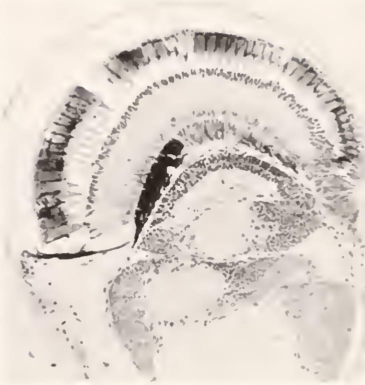
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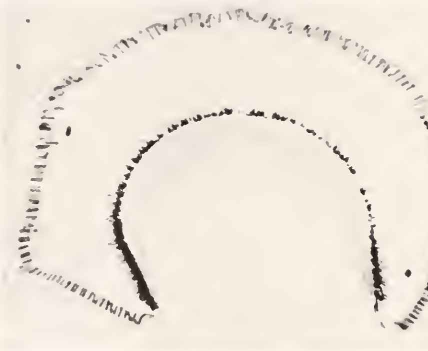
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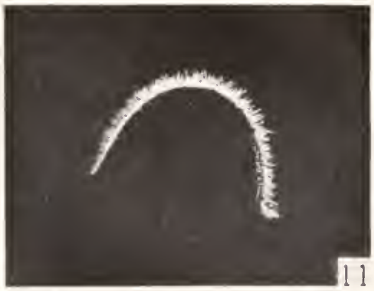
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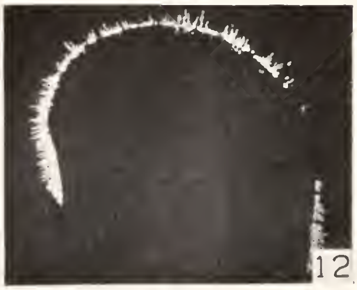
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FIG. 8. Dorsal view of the head of *A. purpurea*, × 2.

FIG. 9. Photomicrograph of a section of the eye of a young *A. purpurea*, × 40.

FIG. 10. Photomicrograph of an unstained section of the eye of a mature *A. purpurea*, × 40.

FIG. 11. Reflecting pigment of the eye of a young *A. purpurea* as seen by reflected light, × 35.

FIG. 12. Reflecting pigment of a mature *A. purpurea*, × 35.

In *H. glacialis* the rhabdomes are entirely lacking. The nuclei of the retinular cells remain, and a few nerve fibers leave their vicinity (Fig. 13). At best the eye of this form can be used only as a light receptor. While not as degenerate as the eyes of certain blind crayfish (Parker, 1890) there is certainly not the necessary visual apparatus for image reception.

EYE PIGMENTS

Unpublished results obtained by one of us from keeping certain decapods in constant darkness for a period of months indicate that there may be a gradual reduction in the amount of screening pigment in the eye. This is perhaps to be expected from the work of Odiorne (1933) on the effects of black and white backgrounds on the body pigments of certain fishes. Is there any correlation in the acanthephyrids under consideration between depth and the amounts of screening and reflecting pigment? In practically all decapod crustaceans from shallow water there are two sets of screening pigments. The so-called distal or irispigment is found around the cones, and in many forms such as *Palæmonetes* (Welsh, 1930) it migrates in and out under the influence of hormonal action (Kleinholz, 1936), forming the pseudopupil which helps to regulate the amount of light reaching the rhabdomes. In certain shallow-water prawns which are distinctly nocturnal in their habits such as *Penæopsis goodei* (Welsh, 1935) and species of *Brachycarpus* and *Rhynchocinetes,* the eyes of which have not been described, the distal pigment forms a collar around the outer ends of the cones and remains in that position in light and dark-adapted eyes. In *S. debilis* the distal pigment is present in an amount comparable to that found in nocturnal surface forms while it is slightly less dense in *A. purpurea* and completely lacking in *H. glacialis.* In specimens of *S. debilis,* light-adapted for three hours, there was no measurable migration of the distal pigment. In specimens of both *A. purpurea* and *S. debilis,* fixed directly from the nets either during the day or night, the distal pigment was always in the extreme outer position.

The second set of screening pigment, the proximal pigment, is found in the retinular cells. In dark-adapted eyes of decapods in general, the main mass is found below the basement membrane, while in the light it moves peripherally to surround the rhabdomes and migrates between the plates. In this position it absorbs a large part of the light which reaches the rhabdomes before it has penetrated to any considerable depth. In all specimens of *A. purpurea* and *S. debilis* taken from the nets during the day or night the proximal pigment never surrounded the rhabdomes and the major portion was in the position characteristic of extreme dark adaptation. In adults of these two species this pigment is present in about equal amounts, but is much less dense than in nocturnal forms found in surface waters. In young of *A. purpurea*, however, the proximal pigment is very heavy in the lateral portion of the eye (Fig. 9). In *H. glacialis* the proximal pigment is entirely lacking as is the distal pigment.

If we assume that the diurnal vertical migrations of *A. purpurea* and *S. debilis* keep these forms in a region of very low but constant light intensity, it would be interesting to know whether the proximal pigment still retains the ability to migrate around the rhabdomes when these forms are subjected to illuminations of high intensity. It was possible to test this point with *S. debilis* and *A. purpurea*. Figure 16 is a photomicrograph of a section of the retina of a specimen of *S. debilis* which had been kept in darkness for three days. Figure 15 is of a similar region of the retina of a specimen kept in diffuse daylight for one hour. During this time the pigment had migrated for some distance peripherally and had surrounded the proximal two-thirds of the rhabdomes. This indicates that the ability of the proximal pigment to migrate still persists even though it may never do so normally in the lifetime of the organism. The proximal pigment of *A. purpurea* is also capable of movement although after prolonged light-adaptation it seldom migrates far into the retinular cells.

The reflecting pigment of decapod eyes is a layer of amorphous guanin which is concentrated around the bases of the retinular cells and acts as a mirror to reflect light back into the rhabdomes (Welsh, 1932). A reflecting or tapetal layer is found in most animals which are active during the night or which live in a region of low light intensity; therefore it is not surprising that this pigment layer is well-developed in the eyes of deep-water decapods.

EXPLANATION OF FIGS. 13–18

FIG. 13. Section of the eye of *H. glacialis* photographed with transmitted light, × 60.

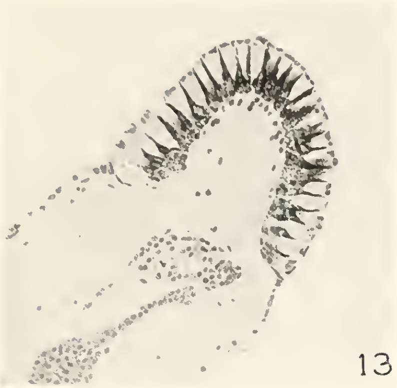
FIG. 14. Section of the eye of *H. glacialis* photographed by means of reflected light, × 60.

FIG. 15. Region of the retinular cells and rhabdomes of a light-adapted eye of *S. debilis*, × 175.

FIG. 16. Region of the retinular cells and rhabdomes of a dark-adapted eye of *S. debilis*, × 175.

FIG. 17. Section of a portion of the eye and the papilla of *O. grimaldii* photographed with transmitted light, × 50.

FIG. 18. The same photographed with reflected light, × 50.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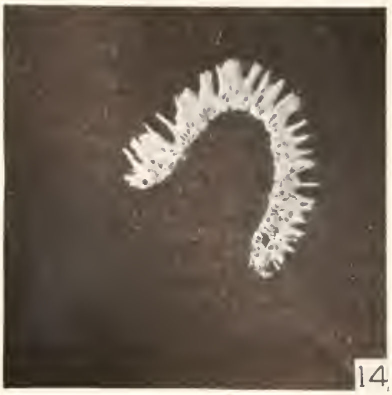
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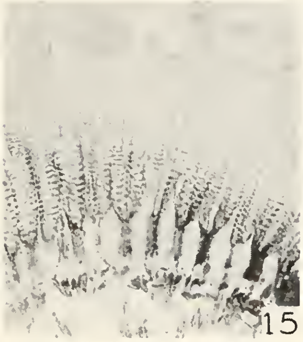
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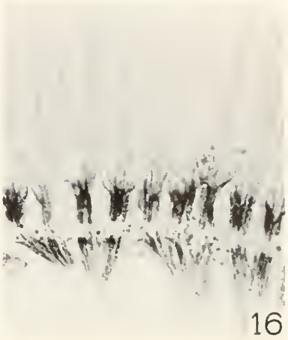
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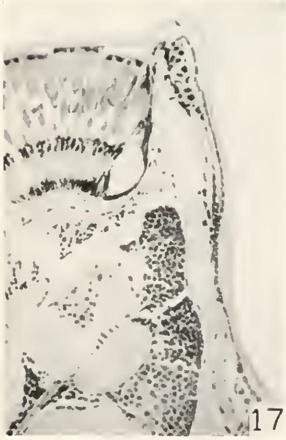
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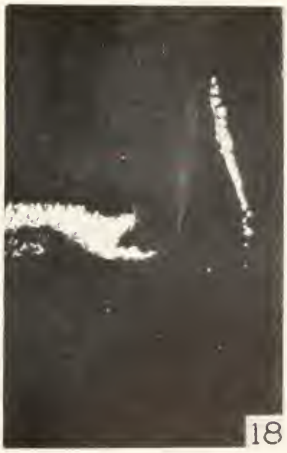
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FIGURES 13–18

The eyes of *A. purpurea* and *S. debilis* have this reflecting layer in addition to the usual screening pigments (Figs. 11 and 12) while *H. glacialis* has only reflecting pigment (Fig. 14). The quantity of reflecting pigment in *H. glacialis* is greater in proportion to the size of the eye than in the other two forms under consideration. It is worth noting that in a series of five species of *Sergestes* which have been studied in a preliminary way there is a rather striking correlation between the amount of reflecting pigment and the depth at which the several forms were taken.

Discussion

The gradual modification of an organ such as the eye as a result of environmental changes, or changes in habit, is one of the fascinating aspects of evolution. A comparison of the eyes of forms which are active at night with those which are active during the day reveals structural and functional adaptations which are among the best examples of the manner in which living material may be modified by external conditions. The degeneration of the eye of cave crayfishes (Packard, 1888; Parker, 1890) is a striking illustration of the disappearance of a useless organ. In the sea we have a gradual reduction in the amount of sunlight which penetrates into the water until a depth is reached, which varies with the locality, at which there is a complete absence of sunlight. The animals of the deep sea have without much question evolved from shallow water forms. Their eyes have become modified depending on the depth to which they have migrated. In regions of low light intensity they are, in general, so changed by an increase in size, loss of screening pigment, and in other ways that they are doubtless quite effective organs of sight. Below the level to which light penetrates some are degenerate and some are completely lacking. On the other hand, some are large and, structurally at least, well adapted for vision or for light reception. This fact still remains as one of the most baffling problems associated with the biology of deep-sea animals. Can there be enough light produced by luminescence to account for the well-developed eyes of some abyssal forms, particularly those living on bottom? This is the question with which most discussions of the eyes of deep-sea animals have ended. It is our hope that if the opportunity remains to continue these studies this question may be satisfactorily answered.

Summary

1. Three species of acanthephyrids have been taken in closing nets in the region of the Sargasso Sea and in slope water near the Gulf

Stream in numbers sufficient so that their vertical distribution is quite accurately known.

Acanthephyra purpurea and *Systellaspis debilis* are found mostly within the photic zone. *Hymenodora glacialis* inhabits a region below that to which sunlight penetrates.

2. The eyes of *A. purpurea* and *S. debilis* are quite similar structurally to the eyes of shallow-water prawns, except that there is less screening pigment.

3. Species of *Systellaspis* and *Oplophorus* possess photophores and the eyes of these forms are larger in proportion to body size than the eyes of those acanthephyrids which lack photophores.

4. The eyes of *H. glacialis* are quite degenerate. The rhabdomes, and both distal and proximal pigments are lacking. The reflecting pigment layer is well developed.

5. Characteristic movements of the proximal pigment of *S. debilis* and *A. purpurea* occur as the result of light adaptation.

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EVIDENCE FOR THE PRODUCTION OF ACCELERATOR AND DEPRESSOR SUBSTANCES BY ULTRAVIOLET RADIATION OF LIMULUS MUSCLE

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The object of this investigation was to determine the causes of the frequency changes after ultraviolet radiation of *Limulus* muscle. Hinrichs and Genther (1930) reported that as a result of exposure to ultraviolet point radiation " in general, an increase in rate of beat was obtained by short exposures of a given region [heart of *Limulus polyphemus*], and a decrease in rate following longer exposures." Guttman (1936*a*) reported that short periods of radiation (3 minutes) from a Cooper-Hewitt Uviarc caused a marked primary increase in frequency and amplitude of the *Limulus* heart.

The heart of *Limulus polyphemus* was excised along with the dorsal ganglion. The rhythmically beating heart was then placed in a finger bowl containing 200 cc. of sea water. Temperature changes were observed by means of a thermojunction. One blackened junction was placed on the ganglion at the level of the sixth segment. As the heartbeat of the *Limulus* is neurogenic (Carlson, 1904 and 1905; and Garrey, 1932), its frequency and amplitude depend on the action of the ganglion. Thus, if the temperature of the ganglion is recorded, it is easy to determine whether frequency changes are attributable to temperature variations of the pace-maker. During the course of this investigation temperature increases caused by the heat of the mercury arc were not responsible for the observed changes. The increases in temperature never exceeded 0.3° C. for direct radiation of the heart and markedly less for the shielded preparations as a result of a 3-minute period of radiation. This temperature change was found to have a negligible effect.

A series of 13 experiments were performed on hearts which were partially shielded. In this series of experiments the excised heart, as stated above, was placed in a finger bowl and a shield (layers of white paper, tin foil, and black paper—the white paper outermost) was placed over segments 3–9; i.e., only segments 1–2 received the radiation. A frequency increase was noted. After a length of time the entire heart was radiated, and finally segments 3–9 were radiated while 1–2 were shielded—see Fig. 1. Frequency increases were always greatest for total radiation, next for segments 3–9, and smallest for segments 1–2.

Another series consisted of 5 experiments, in each of which 2 excised hearts were placed in a finger bowl with the usual 200 cc. of sea water. One of the hearts was shielded and 2–3-minute periods of radiation were given to the unshielded heart. The usual frequency changes (primary acceleration) were observed for the irradiated heart. The shielded heart also markedly increased its frequency. The increase in frequency (expressed in per cent) was usually greater in the unirradiated heart than in the radiated preparation—see Fig. 2. An explanation is suggested below. When hearts which did not beat because of injury to the ganglion during dissection were irradiated for 2–3 minutes, the rhythmically beating hearts (shielded) always exhibited a marked frequency increase.

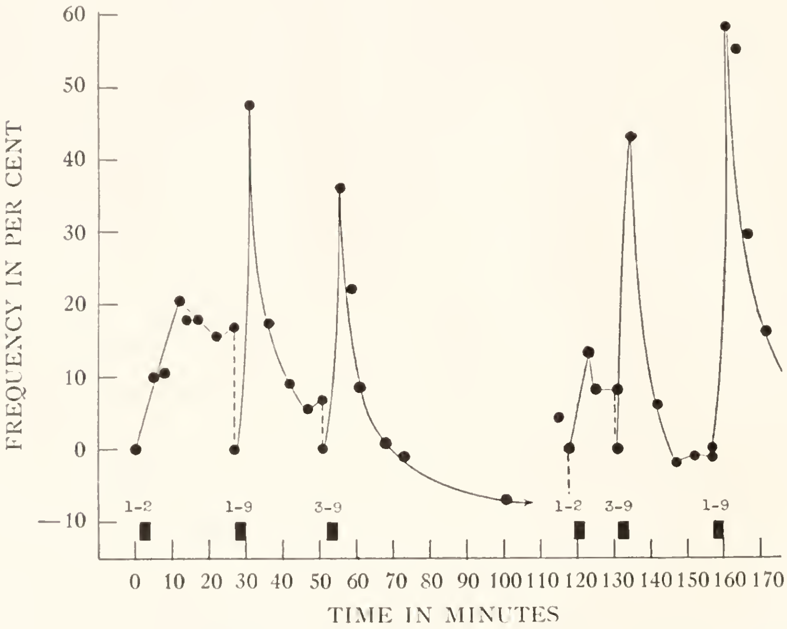

Fig. 1. Curve showing frequency changes of *Limulus* heart after radiation of various regions. ● frequency; ■ period of radiation; 1–2 radiation of segments 1–2; 3–9 radiation of segments 3–9; 1–9 radiation of segments 1–9 (entire heart).

In several series, following the same procedure as above, masses of *Limulus* skeletal muscle (7 experiments) and smooth muscle (intestine) (6 experiments) were irradiated and the heart was shielded. Here, too, the heart frequency increased after short periods of radiation of the various types of muscle—see Fig. 3.

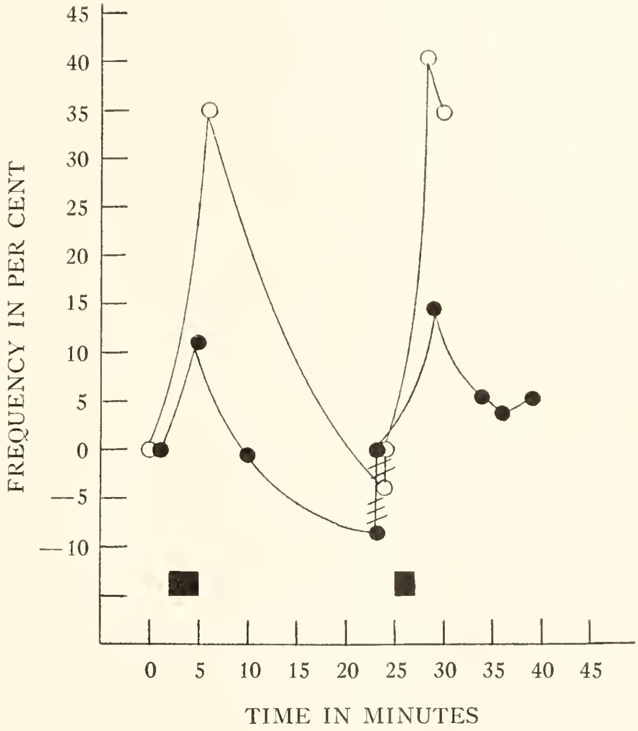

FIG. 2. Curves showing frequency of *Limulus* hearts (1) directly radiated, (2) bathed by sea water which surrounded the radiated heart. ○ frequency of heart in solution which bathed a radiated heart; ● frequency of radiated heart; ■ period of radiation.

Several other series of experiments were performed. It was found that placing a beating heart in irradiated sea water did not induce any frequency change. Radiation of sea water which previously contained unirradiated cardiac, smooth, and striped muscle had no effect on the frequency of *Limulus* hearts. Thus it is concluded that the radiation must be directly on a tissue in order to produce the primary acceleration and that irradiation of sea water is ineffective.

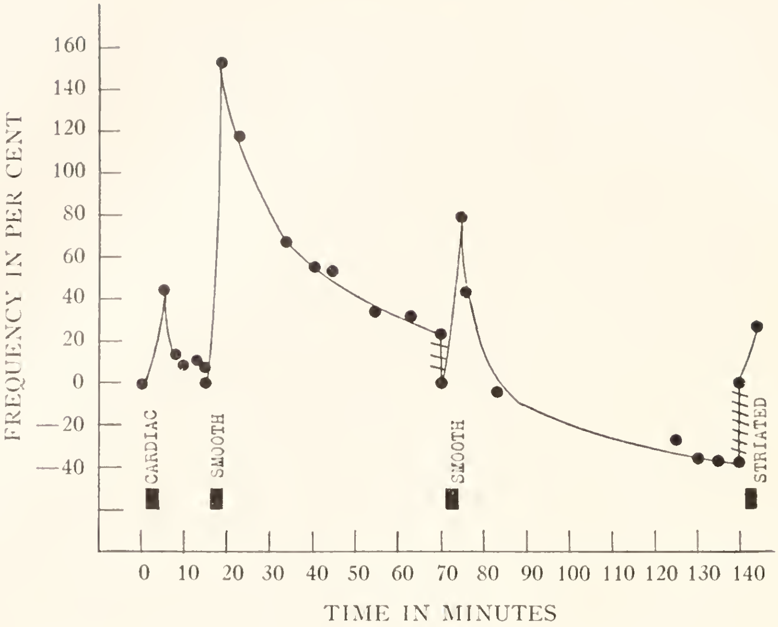

FIG. 3. Curve showing frequency of *Limulus* heart bathed by the sea water which surrounded cardiac, smooth, and striated muscle during radiation. ● frequency; cardiac radiation of cardiac muscle; smooth radiation of smooth muscle; striated radiation of striated muscle.

DISCUSSION

It appears that an accelerator substance may be produced upon irradiation of *Limulus* muscle. Hinrichs and Genther (1930) state that "no change in rate of beat of the entire heart could be demonstrated by direct exposure of the heart muscle itself." It is possible that their negative results can be explained by their use of point radiation. Guttman (1935*a* and *b* and 1936*b*) has presented evidence which indicates the probability of a potassium-calcium shift induced by ultraviolet radiation. This shift, however, probably is not responsible for the frequency changes observed. Guttman (1936*a*) states, "It may be supposed, in the case of the *Limulus* heart, that the primary action of the radiation [ultraviolet] is to increase the frequency of the nerve cell discharges and also to increase the number of active nerve cells; hence the number of nerve fibers transmitting impulses to the myocardium is increased. The former supposition would account for the increased frequency, the latter for the increased amplitude."

It appears that an increase in the frequency and number of nerve cell discharges may be caused by the liberation of some accelerator substance from *Limulus* muscle upon irradiation. It is also well known that nerve is very resistant to radiation and the radiant energy employed could not possibly affect nerve directly. The effect of the substance usually disappears within 20 minutes after the cessation of radiation and then the secondary effect appears (the suppressed frequency and amplitude). It may be that the secondary effect is caused by a second substance produced upon irradiation but the evidence for this is not clear cut and further investigation is necessary. However, this secondary effect does not appear in some hearts which have been irradiated for 3 minutes and an even shorter time of irradiation seems to minimize the secondary effect. In the shielded hearts, stimulated indirectly, the secondary effect appears, apparently due to the diffusibility of the supposed secondary substance. Thus there is the possibility of the production of two chemical substances which are antagonistic in action.

It is a pleasure to acknowledge my indebtedness to Professor W. E. Garrey of Vanderbilt University Medical College, Nashville, Tennessee, for his interest and constructive criticism.

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NOTES ON THE DEVELOPMENT OF GORGODERA AMPLICAVA IN THE FINAL HOST

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INTRODUCTION

Loschge (1785) first reported flukes in the bladder of *Rana esculenta* and Zeder (1800) named them *Distomum cygnoides.* On the basis of the number of testes, Looss (1902) separated the bladder flukes of frogs into two genera, *Gorgodera* and *Gorgoderina,* the former genus possessing nine testes and the latter, two. Bladder flukes of frogs have been reported in North America by Leidy (1851), Bensley (1897), Stafford (1902), Cort (1912), Ingles and Langston (1933), and Ingles (1936). Seven species of gorgoderid worms have been described in North America: *Gorgodera amplicava* from *Rana clamitans, R. catesbeiana,* and *R. pipiens; Gorgodera minima* from *R. catesbeiana* and *R. pipiens; Gorgoderina simplex* from *R. catesbeiana* and *Bufo lentiginosus; Gorgoderina translucida* from *Bufo lentiginosus* and *R. virescens; Gorgoderina attenuata* from *R. catesbeiana* and *R. virescens; Gorgoderina multilobata* from *R. boyli* and *R. aurora; Gorgoderina aurora* from *R. aurora.* Krull (1934), who reported the principal stages in the life history of *Gorgodera amplicava,* found that the clam, *Musculium partumeium* served as the first intermediate host, the snail *Helisoma antrosa* as the second intermediate host, and the frogs *R. clamitans* and *R. catesbeiana* as the final hosts.

The first life history studies on bladder flukes were done by Ssinitzin (1905) who traced the development of *Gorgodera cygnoides, Gorgodera pagenstecheri,* and *Gorgodera varsoviensis.* He found that the cercariæ were of the cystocercous type, that they were produced in bivalve mollusks, and that the metacercariæ developed in the aquatic larvæ of insects. Lutz (1926) indicated the intermediate hosts through which species of *Gorgoderina* might pass in order to complete the life cycle. He found the cercariæ in two small bivalves, *Cyclas* and *Pisidium* or *Sphærium,* and the metacercariæ encysted in the esophagus of odonatan larvæ. The same author also stated that the parasites in the final host, which is some anuran, were often found in the ureters. He observed that "En effet, les conduits efférents du rein contiennent assez souvent les formes jeunes. Dans le *Leptodactylus pentadactylus*, *Gorgoderina permagna* devient presque mûre et *Gorgoderina diaster* a été trouvée pleine d'œufs dans l'uretère de *Pseudis paradoxa*. Chez la *Rana palmipes* il y avait des exemplaires dans la vessie, mais le plus grand se trouvait dans l'uretère." Joyeux and Baer (1934) found three young *Gorgoderina* in the muscles of the ventral body wall of *Rana esculenta*; the worms were not encysted and appeared to be migrating in the muscles without provoking any reaction on the part of the host. They further observed that the genital apparatus was fully functional and that the uterus was full of eggs, some of which had reached the genital pore. These specimens they reported to be morphologically similar to the adult form of *Gorgoderina capsensis* which they described from the urinary bladder of *Rana esculenta*. They concluded that the trematodes may become adult before they reach their definitive habitat, the urinary bladder.

The present paper records the occurrence of *Gorgodera amplicava* in the kidneys of *Rana catesbeiana*, together with a brief description of the trematode, particularly of its reproductive system, in the final host.

Material and Methods

The material for the present study was obtained from the common bull frog, *Rana catesbeiana*, at the Marine Biological Laboratory, Woods Hole, Mass. In routine examination of frogs during the summer of 1936, a number of trematodes were observed in the kidneys. Accordingly, the excretory organs of fifteen frogs were dissected out for further study. No worms were found in the ureters. The kidneys were pressed between two glass slides in order to observe, if possible, the location of the flukes. This method did not prove successful because of the amount of blood present, so the kidneys were dissected, a small portion at a time, until the parasites were located. Twenty-four worms were thus removed, flattened under a cover-slip, and fixed in hot corrosive sublimate. All material was stained in paracarmine, and measurements were made on fixed and stained specimens.

The writer wishes to express his appreciation to Professor H. W. Stunkard for helpful suggestions during the course of the study and in the preparation of the manuscript.

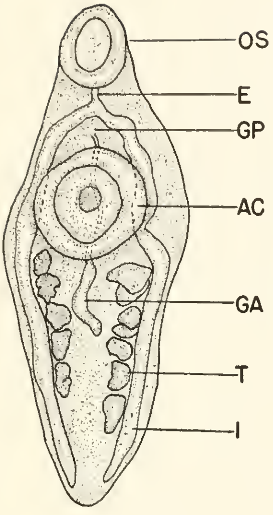
Observations

With but one exception, all of the worms found in the kidneys were sexually immature. In the examination of one frog, a sexually mature worm was observed emerging from a white, cyst-like enlargement on the surface of the kidney. Upon fixing and staining, it was found to be a specimen of *Gorgoderina attenuata* and not a representative of the genus *Gorgodera*. The cyst-like structure possessed an opening at the top through which the fluke probably passed its eggs. This trematode was the only sexually mature specimen found in the kidneys; all the small, sexually immature worms were *Gorgodera amplicava*.

The smallest specimen of *Gorgodera amplicava* from the kidney measured 0.753 mm. in length and 0.255 mm. in width immediately posterior to the acetabulum; the largest one measured 1.68 mm. by 0.45 mm. Neither one was sexually mature. The smallest specimen from the bladder measured 1.76 mm. in length by 0.52 mm. in width. At this stage, the specimens were also sexually immature. Although the increase in size is not significant, comparison of the reproductive organs in the largest specimen from the kidneys with those in the smallest one from the bladder shows the latter to be much more nearly mature.

The series of worms ranging from the smallest individuals in the kidneys to the sexually mature forms in the bladder exhibits a gradually increasing development. In the youngest worms from the kidney, the genital anlagen, with the exception of the testes, appear as a sac-like mass of cells immediately posterior to the acetabulum (Fig. 1). In the next stage (Fig. 2), this mass has increased in size, branched into a right and a left lobe, and has also elongated posteriorly. The lobes then become separated from the common cell mass to form two discrete bodies, the future ovary developing from the left lobe and the vitelline follicles from the right one. At this stage, the vitelline follicles consist of a single mass of cells, later division resulting in the two groups of follicles, left and right, that are characteristic of the adult.

Gorgodera amplicava is definitely protandrous. All of the youngest individuals (stage shown in Fig. 1) taken from the kidneys possessed twelve well developed testes, five on the right side and seven on the left. According to Krull (1934), the testes are at first represented by a dense mass which later becomes separated into discrete organs. Originally, the testicular mass was a part of the common genital anlage, but because of the protandrous tendency of *Gorgodera*, the testes develop before the rest of the reproductive system. It appears that the testicular mass always subdivides into more than the nine parts which are characteristic of the adult. As stated above, the youngest specimens possessed twelve testicular masses; this number was reduced to ten in later stages (Figs. 2 and 3), and finally to nine, the number characteristic of the adult.



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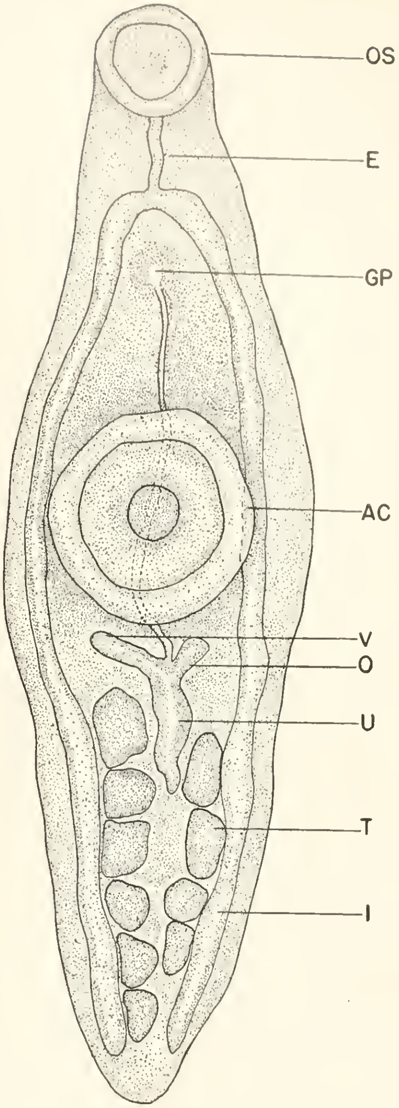

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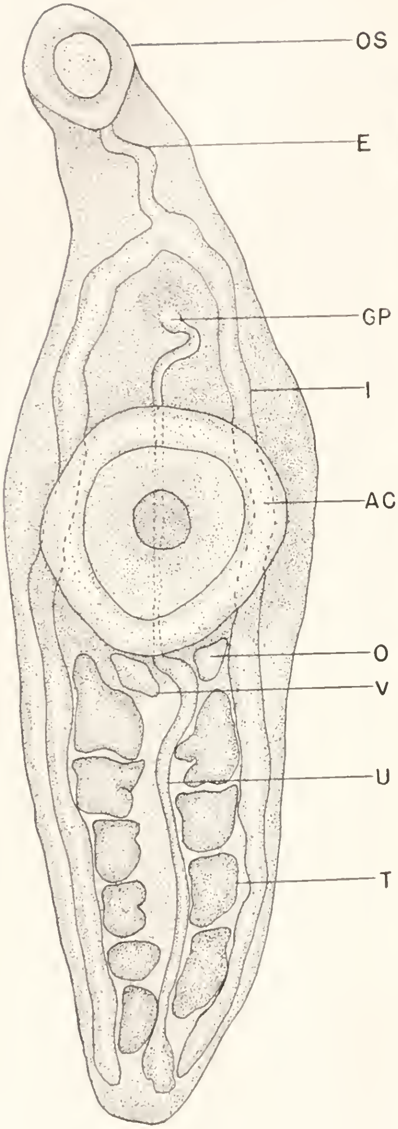
PLATE I

FIG. 1. Smallest form of *Gorgodera amplicava* from the kidney of *Rana catesbeiana*, ventral view. × 85.

FIG. 2. Largest form of *Gorgodera amplicava* from the kidney of *Rana catesbeiana*, ventral view. × 85.

AC, acetabulum.
E, esophagus.
GA, genital anlage.
GP, genital pore.
I, intestine.
O, ovary.
OS, oral sucker.
SV, seminal vesicle.
T, testis.
U, uterus.
V, vitelline follicle.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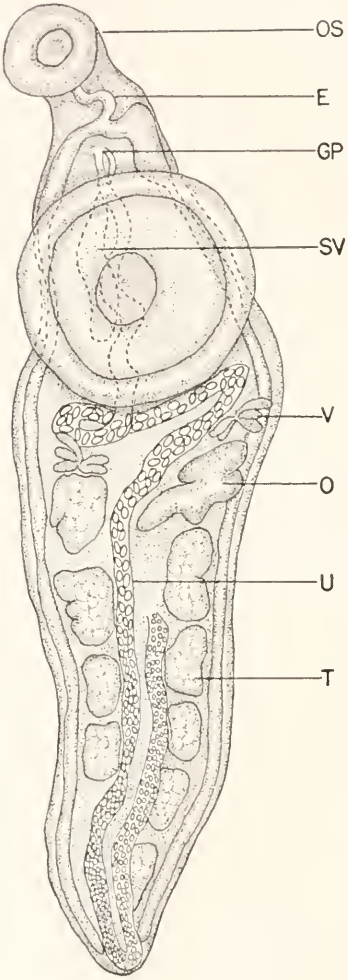
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Plate II

Fig. 3. Smallest form of *Gorgodera amplicava* from the bladder of *Rana catesbeiana*, ventral view. × 85.

Fig. 4. Adult *Gorgodera amplicava* from the bladder of *Rana catesbeiana*, ventral view. × 28.

Discussion

Krull (1934) stated: "In stained and mounted specimens the dense testicular mass shows little evidence of being subdivided into testes, and there is little separation into right and left part until maturity." In the present study, observations on the youngest individuals showed not only division of the mass into right and left components, but also twelve well developed testes; at this time, the female genital complex was as yet undifferentiated. It is probable that the original testicular mass may be subdivided into a variable number of components. In the writer's opinion, after the early division of the testicular mass, there is a coalescence of certain of the testes to form the definitive number.

The observations of Lutz (1926) showed that specimens of *G. permagna* may develop in the ureters of *Leptodactylus pentadactylus* and *G. diaster* in the ureters of *Pseudis paradoxa.* He also found gorgoderid worms in the ureters of *Rana palmipes.* Since these ducts open directly into the cloaca and are independent of the urinary bladder, it appeared that excysted metacercariæ, when they reach the cloaca, may pass either to the bladder or to the mesonephric ducts. Although Lutz himself made the statement: "On distingue *Gorgodera,* a testicules nombreux, de *Gorgoderina,* que n'en a que deux . . . ," it is impossible to determine from his report whether the species *G. permagna* and *G. diaster* are members of *Gorgodera* or *Gorgoderina,* since he referred them first to one genus and then to the other.

The discovery by Joyeux and Baer (1934) of sexually mature specimens of *Gorgoderina capsensis* in the abdominal muscles of *Rana esculenta* is difficult to interpret and introduces further complications in the life history of the frog bladder flukes. It is not impossible that abnormal physiological conditions, resulting from inanition of the hosts, were responsible for migration of the worms to the muscles.

In infection experiments with the definitive host, Krull (1934) fed a number of metacercariæ of *Gorgodera amplicava* to *R. clamitans* and *R. catesbeiana* and later (21 days to 2 months) recovered adults from the bladder. He stated that "On the basis of a rough estimate derived from the infection experiment, it appears that about one-fifth of the number of metacercariæ fed to a frog may be recovered from the bladder upon subsequent examination." About eighty per cent of the specimens fed were not accounted for. No explanation was offered for the disappearance of such a large number of worms, but from the present work it appears probable that a number of them might have been found in the kidney ducts in various stages of development.

Significance must be attached to the fact that developing *G. amplicava* were found regularly in the kidneys. They appeared too often in that location to be merely an accidental infestation, and, furthermore, no sexually mature forms of this genus were found in these organs. It appears probable that the adult *Gorgodera* is too large to migrate up the ureters and for that reason it would be but logical to find only the young stages in the kidneys. On the other hand, the presence of a sexually mature *Gorgoderina attenuata* in the kidney and the fact that *G. attenuata* is much larger than *Gorgodera amplicava* may indicate that it is possible for the adults to migrate to the kidneys. However, the single specimen of *Gorgoderina attenuata,* which was found in the kidney, may have migrated there as a young form and developed to maturity in that location. The fact that no adult specimens of *Gorgodera amplicava* were found in the kidneys and the discovery of a developmental series of worms grading from the smallest in the kidneys to the adults in the bladder, suggest strongly that *Gorgodera amplicava* normally passes a stage of its life cycle in the kidneys before returning to the bladder to become sexually mature.

There seems to be no correlation between the number of worms found in the kidneys and those present in the bladder. In some of the frogs examined, all the specimens were found in the bladder and none were taken from the kidneys; in other instances the reverse was true. In certain frogs, some worms were found in the bladder and others in the kidneys. In every case, however, the specimens from the kidneys were sexually immature while those from the bladder were either adults or individuals in various stages of late development. Yet even here, the smaller forms taken from the bladder were in a more advanced stage of development than those from the kidneys; forms as young as the one represented in Fig. 1 were never found in the bladder. Accordingly, it seems clear that the time of infestation and the rate of development of the parasite may explain the number of worms present in each of the two locations.

SUMMARY

Additional stages in the development of *Gorgodera amplicava* in the final host, *Rana catesbeiana,* are reported. Developmental stages have been found in the kidneys and their ducts and a series of changes in the development of the genital organs is described.

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THE BEHAVIOR OF THE MATURATION SPINDLES IN POLAR FRAGMENTS OF EGGS OF ILYANASSA OBTAINED BY CENTRIFUGING

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The mechanism that carries the maturation spindle to the surface of the egg has never been explained. In most eggs a relatively large mitotic figure develops some distance below the surface. It lies across the primary axis of the egg, perhaps in a more or less oblique position. As it moves or is carried to the pole, it turns on its axis with one pole towards the surface, and during this time the figure as a whole becomes greatly reduced in size. Whether one of the poles of the spindle is destined to come to the surface, or whether it is merely an accident of position that is decisive in this respect is unknown. Of course it is possible if the egg has developed a dorsal-ventral configuration at this time that one rather than the other pole of the spindle is destined to become the polar end of the spindle. If so, this would suggest that the two ends of the spindle may be alike, and the constitution of the egg is the factor determining that one of them becomes the polar end of the spindle. On the other hand, if the two poles of the spindle are in some respect different, its orientation may depend on the mitotic figure rather than on the protoplasmic field. This problem has seldom been discussed in the embryological literature in the absence of any real data that would give information on the situation.

The experiments that follow throw little light on the question but they do have a bearing on the nature of the mitotic figure in relation to the peculiar division that leads to the unequal division of the egg when the polar bodies are given off. For instance, if at the time when the polar spindle is at the surface, it should be carried into the interior of the egg, will it then move again to the surface, or failing to do so, will it bring about an equal or an unequal division of the egg? Again, what will happen if the polar spindle is driven by centrifugal force into the middle of a fragment of the egg? Will it bring about an equal or an unequal division of the fragment when the second maturation division is due? Both of these questions are answered by the following experiments.

The Technique Used to Get Large and Fairly Uniform Fragments of the "Top" of the Egg

The procedure is simple but must be adhered to strictly. Enough sea water is added to ground-up commercial gum arabic to cover it. Heated in a test tube a thick solution, that flows slowly, results. This is practically a saturated solution. A few drops of this solution are put into the bottom of an ordinary centrifuge tube, and covered with about 10 cc. of sea water. The tube is then centrifuged for a few minutes to get air bubbles and detritus out of the gum. When the eggs are added, or just before, a little of the surface of the gum is mixed with the supernatant sea water. This gives a gradient. The eggs come to lie just above the harder gum in this gradient where they remain during the centrifuging. If the gum is too thick, or if a surface is formed between it and the sea water, the eggs resting on the surface are more difficult to break apart. When the conditions are right the " bottoms " of the eggs are driven into the gum, and the tops come to lie in the gradient of gum or in the sea water. Under these conditions there is no crowding of the eggs which lie in a single layer with the pole upwards, or centripetally on the machine. After centrifuging, the eggs are carefully sucked out of the tube with a large pipette, so as to disturb them as little as possible, and put into a dish of sea water. Some of the gum comes out with the tops but does not interfere with the development of the fragment unless too much is present. By agitating the dish gently the eggs separate from any gum adhering to them. The tops are elongated to various degrees but soon after removal tend to contract and often become more or less round. The bottoms, that are driven into the thicker gum, absorb water and die. A few may escape, and these become rounded or oval. It is curious that while all the tops remain alive, nearly all the bottoms become swollen and die. In order to keep a time record, a few eggs are kept outside, and their development recorded for each set. In this way the condition of the eggs at the time of centrifuging and later is obtained. Without this information the results would not be satisfactory.

The Division of Polar Fragments, "Tops," When the Second Maturation Spindle is Present

Since the results with the second mitotic figure are more striking, and under better control, they may be first described. A general statement will give the essential points. When the eggs have been properly centrifuged (see below), at the time when the second antipolar lobe is about to appear or has begun, the larger tops are elongated about once and a half or twice as long as wide. A whole egg about to constrict apart is shown in Fig. 2, and a top from the same lot in Fig. 1. Stained preparations show that the chromosomes of the second maturation division have been driven, as a rule, into the interior of the top. The whole mitotic figure is moved inwards. In the course of half an hour or longer a constriction appears, generally near the middle of the top. It slowly narrows, and the piece is divided into two parts that are generally equal or nearly so. Stained preparations show that two reconstituted nuclei lie, as a rule, one on each side of the division plane. Earlier stages show an anaphase spindle at the constricting level. The time relations show that this division corresponds to the second polar spindle stage. It is somewhat delayed, however, but does not overlap the time when the first cleavage is due. Moreover, that this division is not a cleavage division is shown by the later history of the tops. When the cleavage of the normal eggs takes place, about an hour after the above event, a protoplasmic division occurs in only one of the two halves of these tops, as shown in Figs. 17, 18, 27. This half is the one in which the sperm nucleus comes to lie. Most often the division takes place in the more polar half, but occasionally in the other half, depending on which half contains the sperm nucleus. Stained preparations show, in fact, that after centrifuging the sperm nucleus may lie at any point along the elongated top, and it is largely a matter of chance in which half it comes to lie, but since more often the more polar half of the fragment divides it is obvious that this half more frequently contains the sperm nucleus. Whether there is any advantage in calling the dividing half an egg, and the other half a giant polar body will be discussed later. The essential point is that the polar spindle, when driven into the interior of the fragment, divides the fragment into equal parts, under certain conditions to be described. There were twelve experiments carried out at the second polar spindle stages and in each a few, or in some many, tops divided by means of the second polar mitosis. A few samples will suffice to cover the results.

(1) The second lobe was nearly due when the eggs were put into the centrifuge, and the second lobe began while the eggs were in the machine. The eggs were centrifuged at 1850 r.p.m. for 5 minutes, then at 2500 r.p.m. for 10 minutes, and at 2820 r.p.m. for 5 minutes more. The tops were about 3 times as long as broad, but became shorter when removed to sea water. Eighteen minutes later a top is drawn in Fig. 1. In comparison a whole egg is drawn in Fig. 2. A few tops were dividing 6 minutes later (Figs. 3, 4, 5). Stained fragments at the time of removal from the centrifuge showed a metaphase plate in the stained

area of the tops, and one polar body near the oil. Later some tops had dividing nuclei at or in the constriction.

(2) Eggs were centrifuged for 7 minutes at 1850 r.p.m., then for 12 minutes at 2500 r.p.m. The first lobe appeared in the control while the eggs were on the machine, and the second lobe 4 minutes after the eggs were taken off. One hour after removal four tops constricted and divided, and 40 others did not. Thirty minutes later most of the latter divided into two equal cells at the same time that the normal controls were cleaving. Here the eggs were centrifuged too early perhaps for the second spindle to be effective, and only a few were divided by it.

(3) Eggs in which the second lobe was just beginning were centrifuged for 7 minutes at 1850 r.p.m., and then for 10 minutes at 2920 r.p.m. A few minutes later, when removed, the tops had separated and were elongated, as shown in Fig. 6. They were killed two minutes later, and stained (Figs. 34, 35, 36). Some of the eggs showed the metaphase plate of the second polar spindle in the center of the top; others near the surface in the oil field. Sixteen minutes after removal the tops were about twice as long as broad; i.e., they had shortened and rounded only to a slight extent. Thirteen minutes later some were more rounded, others were elongated and more of these showed a constriction in the stained zone. Killed and stained two minutes later, some of the tops with a constriction showed the polar spindle in metaphase near the constriction (Figs. 37, 38, 39); others, not constricted, showed an anaphase spindle in the oil field (Figs. 40, 41); and some had divided into two nearly equal parts with a nucleus in each part near the constriction (Figs. 42, 43).

(4) Another set was centrifuged four minutes after the second lobe had begun to appear for 8 minutes at 1850 r.p.m., then for 10 minutes at 2820 r.p.m. When removed the tops were (after three minutes) about one and a half to two and a half times as long as broad (Figs. 7, 8). Sixteen minutes after removal the 86 tops were about one and a half times as long as broad. Eight minutes later many tops were slightly constricted, and 2 minutes later most of them were dividing. Those killed two minutes after removal from the machine showed a plate of chromosomes in the stained zone (Figs. 44, 45, 46). The tops, killed two minutes after the constriction appeared, were dividing (Fig. 47); often these constrictions were deeper at one side (Figs. 48, 49, 50). In all cases a nucleus was present on each side of the constriction, and the sperm pronucleus was often seen in the polar half. Four other sets gave results similar to the foregoing.

(5) A set of eggs was centrifuged, at the time when the second lobe was just appearing, at 1850 r.p.m. for 5 minutes, continued at

2820 r.p.m. for 10 minutes, then at 3200 r.p.m. for 2½ minutes. The tops were left in the tubes 5 minutes longer and then transferred to sea water. At this time about half of the tops were about twice as long as wide; a few others were longer with a constriction near the middle, and the rest were oval or pear-shaped. Two of the living tops are drawn in Figs. 9, 10. The larger tops were dividing 13 minutes later (Figs. 11, 12, 13). At this time the second lobe had gone back in the control. The controls did not divide until 32 minutes later. Thus the division of the tops by the second spindle was a little delayed compared with the time of extrusion of the second polar body of the normal control eggs, but took place half an hour before cleavage of the latter. Sixteen minutes later all of the 56 tops had divided (Figs. 14, 15). An hour and a half later one of the two halves was dividing or divided (Fig. 16, 17, 18). The other half was undivided. Some of the eggs had been preserved soon after removal from the machine. A plate of chromosomes (the mitotic figure for the second maturation division) lay in the middle of the stained zone (Fig. 51). Other tops were preserved after the maturation division (Fig. 52), and others after one of the two halves had divided (first cleavage, Fig. 53). The other half contained a nucleus, but was undivided. The former half presumably contained the sperm nucleus.

(6) This set of eggs was centrifuged at the time when the second lobe was about one-third out at 1850 r.p.m. for 5 minutes, then at 2970 r.p.m. for 12 minutes. The tops were off, and were left in the tube for an hour. When removed most of them were elongated and many constricted (Figs. 19, 20, 21, 22). Half an hour later both halves were more spherical (Fig. 23) and remained in this condition for 50 minutes (Figs. 24, 25, 26), when one of the halves began to divide, almost always the polar half (Figs. 27, 28, 29). Eggs killed 6 minutes later showed a variety of conditions; some were still divided into two equal parts (Figs. 54, 55, 56, 57); two of these had a nucleus in each half; one had a division figure (anaphase stage, Fig. 56) in one half and the chromosomes irregularly dispersed at the division plane; one (Fig. 57) had a bridge of chromosomes across the division plane. Two tops are drawn in Figs. 58, 59, in which one half has cleaved into two cells, the other half has a small group of deeply stained chromatin. Two others (Fig. 60) have a line of chromosomes extending across the division plane. One, Fig. 61, has a small cell with a large nucleus in the bridge between the larger halves, and a dark mass of chromatin in one half which may be either the sperm nucleus or a part of the mitotic figure.

The outcome of these experiments shows clearly that the mitotic figure of the second polar spindle may divide a fragment (itself about half the size of the egg or a little larger) into equal or nearly equal halves, provided the top is elongated at the time of division. This means probably that the normal division, at the second maturation, into a minute polar body and an egg is not due to any pecularity of polarity of the mitotic figure, but to its location on the surface of the egg. On the other hand, if the second spindle is driven into the whole egg, and the egg becomes spherical, it fails to divide the egg, unless, as previous work has shown (Clement, Morgan), the egg is elongated at this time.

THE FIRST POLAR SPINDLE IN "TOPS" OBTAINED BY CENTRIFUGING

In order to find out whether the first polar spindle could also divide the tops into equal parts, a number of sets of eggs were centrifuged at different stages in the development of the spindle. The results were negative. It was found that the spindle remained at the surface of the egg, or else moved there, and gave off a polar body.

(7) A capsule, just laid, was opened, and the eggs centrifuged at once. A few eggs were preserved before centrifuging. These showed the chromosomes in metaphase in the polar hemisphere. After 24 minutes on the machine tops separated from bottoms. They were much elongated at first. Six minutes later they were still elongated (Fig. 30), but not so much so as at first. One of the bottoms is drawn in Fig. 31. The tops became spherical and polar bodies were given off in or near the oil caps. None divided into equal parts at the time of formation of the first or of the second polar body. Later they cleaved into 2 cells when the first (delayed) cleavage was due.

(8) Another set of eggs from a capsule just laid was centrifuged, in all 47 minutes; 5 minutes at 1850 r.p.m., 10 minutes at 2970 r.p.m., 6 minutes at 3450 r.p.m., and 26 minutes at 2970 r.p.m. When removed the tops were greatly elongated (Fig. 32). The first polar body had not been given off. Twenty-four minutes later a polar body appeared in the oil in some tops, and during the next 20 minutes appeared in more tops. The first lobe of the control appeared 16 minutes after beginning centrifuging; and the second when the tops were still on the machine. Sixteen minutes later, when the tops were more contracted, none had divided and several at least had two polar bodies. Most tops were constricted at this time, but no division occurred. There were, however, several fragments (in which no polar body was seen), which were deeply constricted, but as these did not contain the oil field they might be called middles. Later, after 26 min-

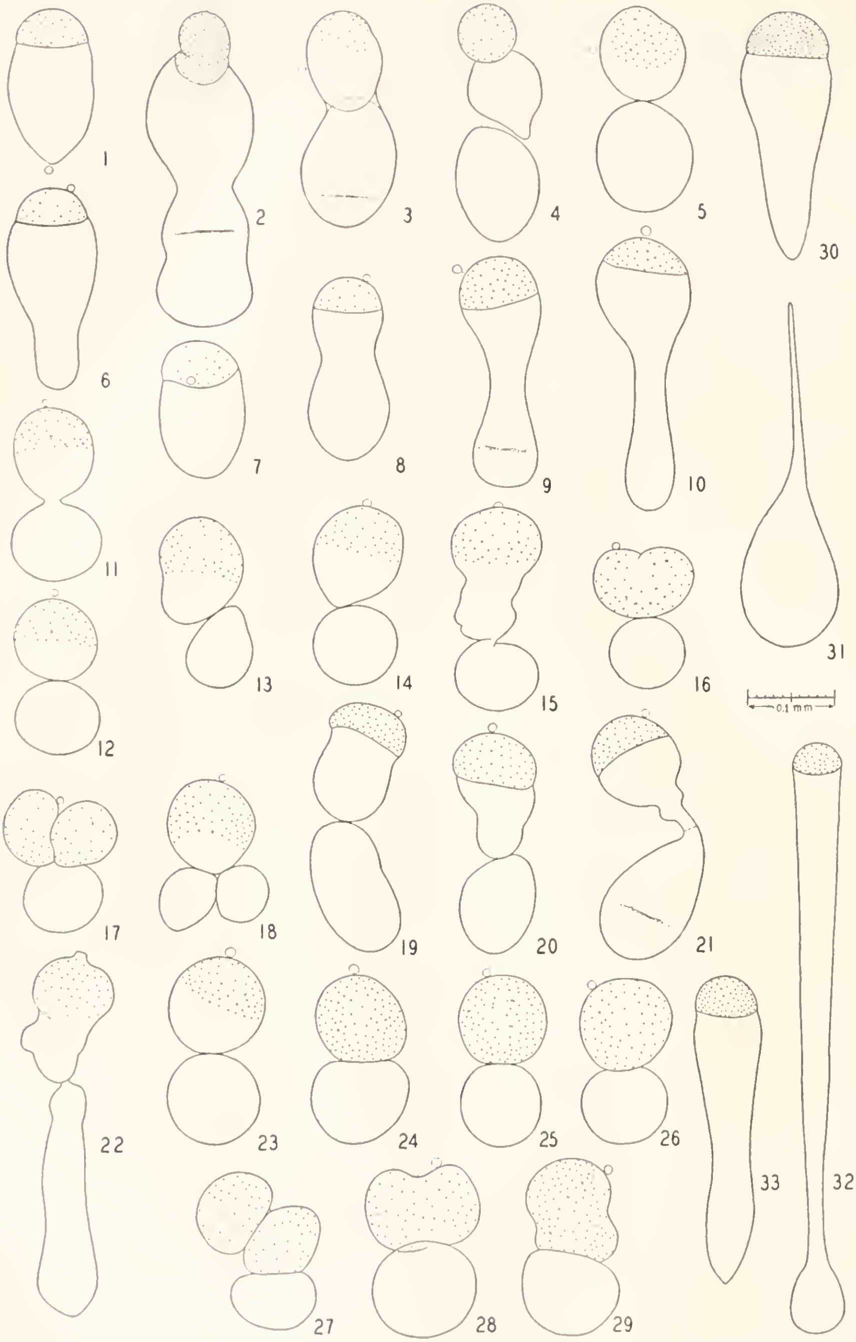
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PLATE I

FIGS. 1–33. Tops of eggs (except Figs. 2 and 31) separated over gum arabic. These are all drawn to the same scale from living material. For details see text.

utes, one third of these divided into equal parts. The control did not divide at this time so that these may represent either the first or more probably the second polar mitosis. In two of these only one of the equal cells divided again, as is the case of tops divided by the second polar mitosis. Some of the tops of this set, killed at the time of removal from the machine (Figs. 63, 64), showed the polar chromosomes in the oil cap, possibly in anaphase. The sperm nucleus was deeper in the protoplasm. If, in some of these tops, the oil with the polar chromatin had been driven off, the middles with the sperm nucleus might be expected to divide at the cleavage stage, but I have no record of such cases.

(9) Another set of eggs, centrifuged 42 minutes before the first polar body was due, was not divided by the polar spindles.

(10) Another set was centrifuged one hour and 15 minutes before the first lobes appeared. Three tops divided into two parts at the time of the second spindle, but 86 did not.

(11) Another set centrifuged for 19 minutes, one hour before the first lobe, at 1850 r.p.m. for 5 minutes, then at 2970 r.p.m. for 12 minutes, and at 2970 r.p.m. for 2 minutes. When removed the tops were much elongated (Fig. 33), and no polar bodies had been given off. Seventeen minutes later polar bodies appeared in the oil, or at the side. Eighteen minutes later most of the tops were spherical; some were still elongated. None had divided 35 minutes later. Tops killed when removed from the machine showed a metaphase plate in the polar part of the stained area (Fig. 62). Fifty minutes later two polar bodies were at the side of some tops, but none showed chromatin in the middle of the stained zone, i.e., the second spindle had come to the surface.

The failure of the first spindle to divide the tops, as does the second spindle when driven from the surface, may be due to failure to have centrifuged at the right stage, but this seems improbable since a fairly wide range of times was tried. The failure may be due in part to the first spindle being driven into the oil region or near the surface, rather than into the protoplasmic zone. In other cases when the spindle was present in the top it moves to the surface of the oil or to the side of the top, and gives off one or two polar bodies there. It seems then that, compared with the behavior of the second spindle, the condition of the egg is such that the first spindle comes to the surface again while the second does not always do so. In what respect the conditions are different is impossible to state. The difference has nothing to do with the attachment of one pole to the surface in one case and not in the other, since in *Ilyanassa* it is the whole polar spindle that is carried

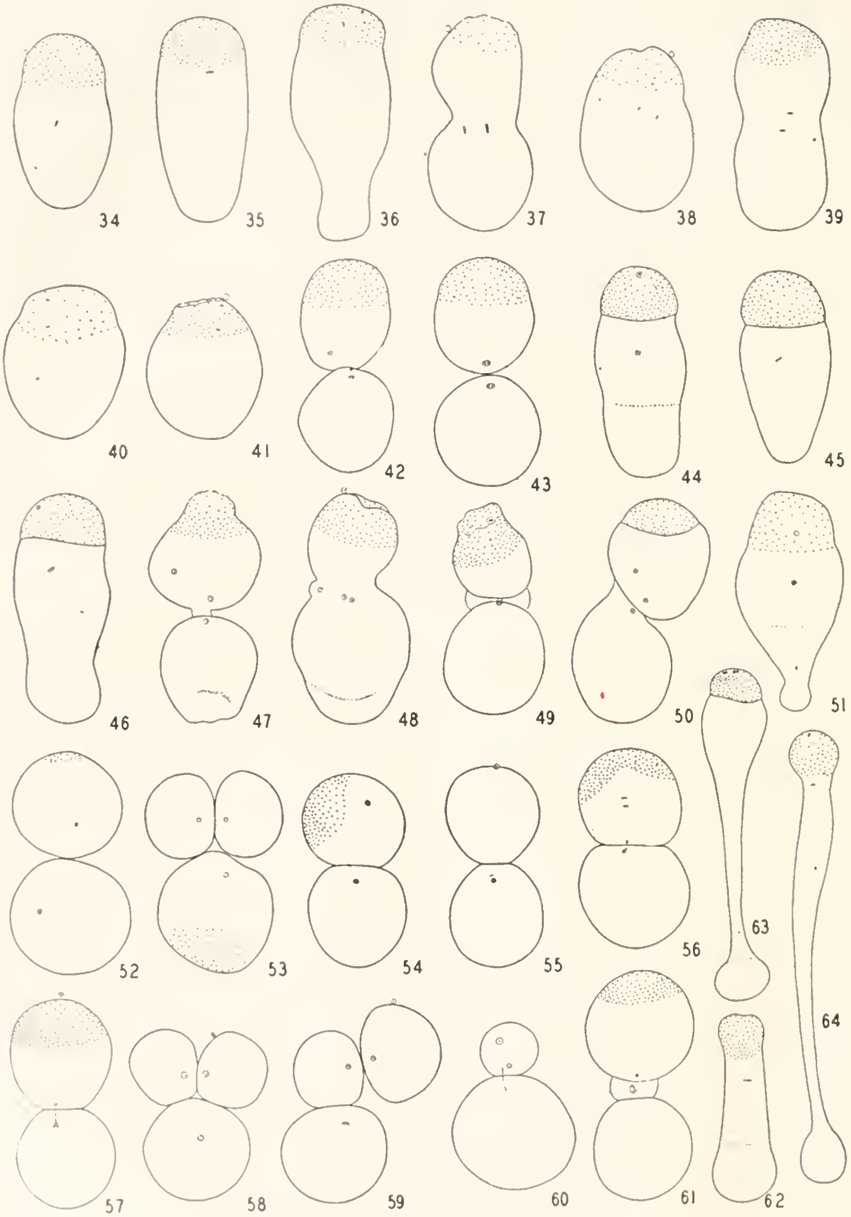
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PLATE II

FIGS. 34–64. Tops of eggs drawn from preserved material. These are not on the same scale as Figs. 1–33. They were smaller, due to shrinkage in preserving and mounting in balsam, and were enlarged and reduced on a different scale from those in Plate I.

into the interior, or else it is not moved at all. Neither a short, nor a long time in the centrifuge seemed to affect the results. It seems improbable that the two spindles are in themselves different with respect to their power of dividing a fragment. The failure of the first spindle to divide the top in these experiments seems rather to be due to a failure to drive it or hold it in the middle of the fragment. Under other conditions it may be found possible to do this.

CONCLUSIONS

When these results are compared with those obtained by Morgan (1935) and Clement (1935) where the eggs of *Ilyanassa* were elongated in the centrifuge but the tops have not pinched off, it is evident that in both it is the second spindle that caused the division of the egg or of the top, if at the time the whole egg or the top has retained to some extent its elongated shape. If the top contracts into the egg, the egg is not divided by the polar spindle, even although the second polar body is not extruded. It seems that the stretching of the egg is correlated with the division and is a contributing factor. I have discussed elsewhere whether there is any advantage in speaking of one of the parts after the division of the second spindle as a giant polar body and the other as the egg. It is even less apparent in the case of these tops that anything is to be gained by such a comparison. The main point is, I think, that the results show that the second spindle, removed from the surface by centrifuging, may divide the egg or fragment of the egg into two equal parts, provided the egg or the top is still elongated at the time of division. In the case of *Crepidula,* Conklin ascribed the formation of the giant polar bodies to the forcible removal of one daughter group of second polar body chromosomes from the other group. In *Ilyanassa,* on the contrary, the division is brought about by the removal of the whole mitotic figure from the surface, and in this respect it may appear that the division that follows may be more nearly compared with the maturation division that gives rise to a polar body. It would seem that the eggs of *Crepidula* and of *Ilyanassa* behave differently on the centrifuge and consequently their subsequent behavior is different.

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BUDDING AND LOCOMOTION IN THE SCYPHISTOMAS OF AURELIA

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The Scyphozoa have three principal methods of reproduction: (1) sexual reproduction by means of gametes formed by the free-swimming medusæ; (2) lateral budding by the scyphistomas or polyps which develop from fertilized eggs; and (3) strobilization—a sort of transverse fission by which a scyphistoma gives rise to a series of young medusæ. The studies here reported concern the second of these methods of reproduction; namely, lateral budding. They concern also the closely related phenomenon of locomotion by means of " sterile buds " or pedal stolons.

Lateral budding in a scyphistoma was described one hundred years ago by Dalyell (1834), who also saw strobilization (1836) and noted that after giving off the young medusæ (ephyræ), the basal portion of the strobilizing polyp (strobila) returns to the vegetative or scyphistoma stage. Thus, according to Dalyell, the scyphistoma or " Hydra tuba," as he termed it, is a perpetual condition, able to maintain itself indefinitely by budding. Hérouard (1908) has described budding and locomotion (" métrotropism ") ; while a fuller account is given by Pérez (1922) ; and more recently by Halisch (1933). The present account covers somewhat the same ground, but undertakes by observation and experiment, to discover the causal relations involved. In short, we shall attempt to be explanatory.

MATERIAL

The polyps used were presumably those of *Aurelia*, and were obtained in great abundance from the underside of an old float in a slough not far from Pacific Grove, California. They were obtained also from the hulls of destroyers which had lain at anchor for four years in San Diego Bay. I wish to acknowledge my indebtedness to the two California institutions whose guest I was while making these studies: the Hopkins Marine Station of Stanford University, located at Pacific Grove, and the Scripps Institution of Oceanography of the University of California, located at La Jolla.

A scyphozoan polyp (or scyphistoma) consists of two germ layers, ectoderm and entoderm, separated from one another by a thick layer of gelatinous mesoglœa. Its body may be divided for purposes of description into two parts: (*a*) a body portion proper, terminating distally in an oral disc (peristome) bordered by a circle of tentacles and surrounding the mouth. We may call this terminal portion of the body, for want of a better name, the "hydranth." The lips of the mouth project somewhat, as a proboscis. The body is partially constricted internally by four longitudinal folds of entoderm, the gastric ridges (tænioles). (*b*) Below the body is the stalk, a simple cylinder which ends in a base of attachment. Commonly there is a small amount of transparent secretion about the base; but this is too delicate to serve for attachment.

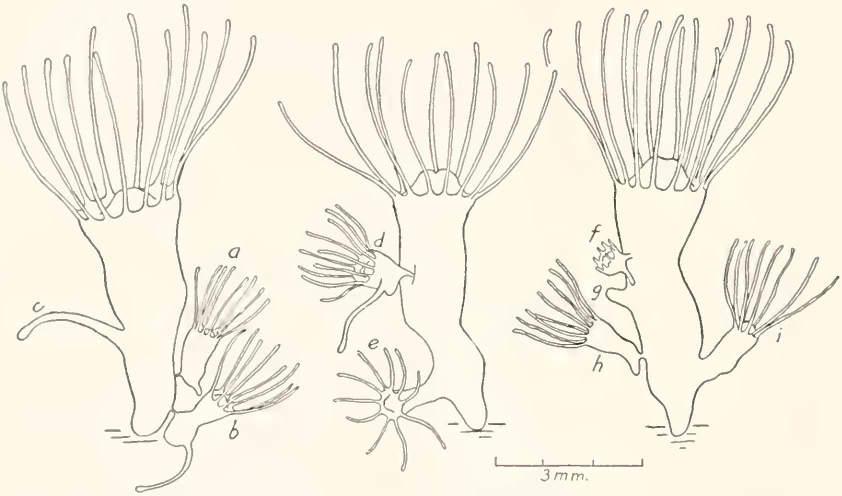

FIG. 1. Scyphozoan polyps (*Aurelia*) showing buds and pedal stolons. *d* and *f* are typical "fig-type" buds. *c* and *g* are pedal stolons. *c* is a "hydra-type" bud. *a, b,* and *h* are intermediate.

DESCRIPTIVE

Buds and Pedal Stolons

Buds and stolons develop from the wall of the lower body and stalk. They may be classified as of four principal types; although variation within limits, rather than conformity to rule, is the rule in scyphistomas.

(1) The *fig-type bud* (Fig. 1, *f* and *d*) is an outgrowth of usually the lower body or upper stalk. Typically it is compressed from side to side, and it soon begins to constrict away from the side of the parent.

A mouth and circle of tentacles develop on the side of the bud which is towards the parent's tentacles, while a tendril-like stolon grows out on the side of the bud away from its attachment to the parent. Sometimes the hydranth forms before the stolon appears; sometimes the reverse is the case; but most frequently the tentacles and stolon grow out at about the same time. In the course of a few days the stolon elongates, attaches by its tip, and contracts; thus it draws the bud away from the parent (Fig. 2). A narrow strand of tissue composed of ectoderm only, remains for a time to connect the bud to its place of origin on the side of the parent. Budding of this sort has been described by Perez and Halisch.

(2) A second type of outgrowth is the *pedal stolon* (Fig. 1, *c* and *g*). This is at first a blunt cone, which becomes more and more acute until it is an elongated tendril of nearly uniform cross-section. Pedal stolons grow almost entirely from the upper stalk portion of the polyp. As a rule they are at first directed outward and upward, that is, away from the polyp's base. Shorter stolons which attach rather promptly may grow downward from the lower part of the stalk, especially in polyps which have been torn loose from their place of attachment.

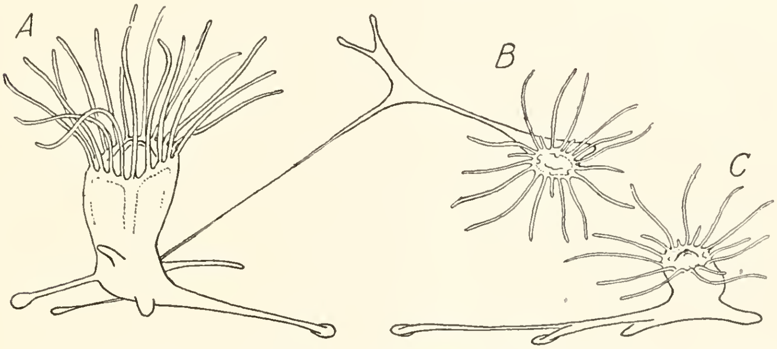

Fig. 2. Locomotion of buds. *A* is an adult polyp with older and younger stolons and from which a bud, *B*, has recently migrated. *C* is another bud migrating.

(3) An intermediate type of outgrowth is the *stolonic-bud* (not figured). This is similar to the pedal stolon in its manner of origin, and usually forms in about the same position. Its distal portion is in fact a pedal stolon; but as it grows outward, its proximal portion draws to itself a more than usual amount of the body wall of the parent polyp. This gradually constricts away from the parent, and as it does so, it forms a mouth and tentacles on the side of the bud near its proximal

end. Sometimes two hydranths are formed side by side, instead of one. The stolonic bud differs from the fig-type bud in that the development of the hydranth and the process of constriction away from the parent do not occur until after the stolon has grown out, and even after it has attached.

(4) The fourth sort of outgrowth is the *hydra-type bud* (Fig. 1, *i*, but especially *c*). This is a cylindrical outgrowth, usually from the lower stalk. Its distal end forms a mouth and circle of tentacles. It does not soon form a pedal stolon, but remains attached to the base of the parent for a long time. Indeed, it may grow to be the size of its parent, with the result that small colonies of several individuals are sometimes formed. Ultimately, however, the buds separate by the formation of pedal stolons on the parent or on the bud, above the place of union. The stolon, after attaching and contracting draws the bud and parent apart. When the stolon is on the parent, the body of the parent may be drawn away, leaving the bud attached to the substrate by the old base of the parent. Such a method of separation is described as of regular occurrence in a scyphistoma of unknown genus (Renton, 1930).

The day-by-day history of the fig-type buds, the sequence of tentacle formation, and the descriptive aspects of locomotion by means of pedal stolons have been given by Pérez (1922) and independently by Halisch (1933).

Locomotion

The Method of Locomotion

Pedal stolons serve two purposes: for locomotion and to provide new bases of attachment. The first process is seen to best advantage in the migration of buds away from the parent (Fig. 2). The stolon grows out on the side of the bud away from the parent's body. It then attaches to the substrate, contracts, and so draws the bud after it. Then another stolon grows out on the side of the bud away from the parent; and this in turn lengthens, attaches, and contracts. Thus step by step the bud moves from its place of origin. After perhaps six steps, the stolons appear less frequently and in less organized fashion; and although change of location still takes place, forward locomotion in one direction is not so consistently observed.

Even in adult polyps, however, forward locomotion in one direction is occasionally seen (Fig. 3). A number of polyps were removed from the oyster shells to which they were attached, into a dish of sea water. Individuals were then chosen which showed neither buds nor stolons, and these were placed in individual dishes. Twenty-four hours later most of them had developed stolons, and again twenty-four hours later most of them had attached. (Possibly isolation favored the formation of stolons, although this is not proven.) Careful drawings were made of certain individuals from day to day; and it was found that some sent out two or more stolons, either simultaneously or in close succession, which thus anchored the polyps to one spot; while the majority formed stolons one by one, roughly at two-day intervals. There was in some instances a tendency for stolons to form successively on the same side, so that a more or less directed locomotion took place.

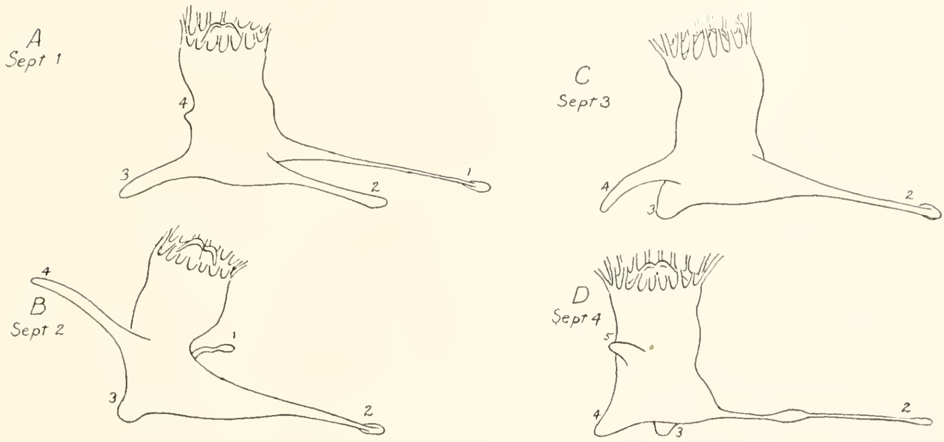

FIG. 3. Locomotion of an adult polyp. The same polyp was drawn on four successive days. The pedal stolons are numbered from 1 to 5 in the sequence of their formation.

"Life History" of a Pedal Stolon

Locomotion by stolons will become clearer if we consider the "life history" of a single stolon (Figs. 3 and 4). (*a*) The stolon begins as a blunt, conical projection from the upper stalk portion of a polyp (Fig. 3*A*, stolon 4). (*b*) It then elongates and pushes outward and somewhat upward away from the base of the polyp (Fig. 3*B*, stolon 4). It continues to grow in length until it has become a tendril-like process composed of an ectodermal sheath with a solid entodermal core, free to wave to and fro in the moving water. Indeed, it apparently has some movement of its own. (*c*) When fully elongated the tip of the stolon (ectoderm) develops glandular cells and becomes adhesive. Coming into contact with some surface it attaches (Fig. 3*C*, stolon 4). (*d*)

Contraction follows immediately, and with so much power that the entire polyp is pulled toward the new point of attachment (Fig. 3*D*, stolon 4). (*e*) The stolon now becomes for a time molded over into the base of the polyp; its attached tip becomes the foot (Fig. 3*D*, stolons 4 and 3). (*f*) In due time another stolon forms, elongates, attaches, and contracts; thus drawing the polyp onward. The old stolon (the base) is thus stretched out into a thread composed almost solely of ectoderm, which for a time retains its attachment to the substrate (Fig. 3*A* to *D*, stolon 2). (*g*) Soon, however, the old stolon breaks from its attachment, and is withdrawn into the side of the stalk of the polyp, usually with some debris adhering (Fig. 3*A* and *B*, stolon 1). The entire history of a pedal stolon thus suggests strongly the behavior of a pseudopod

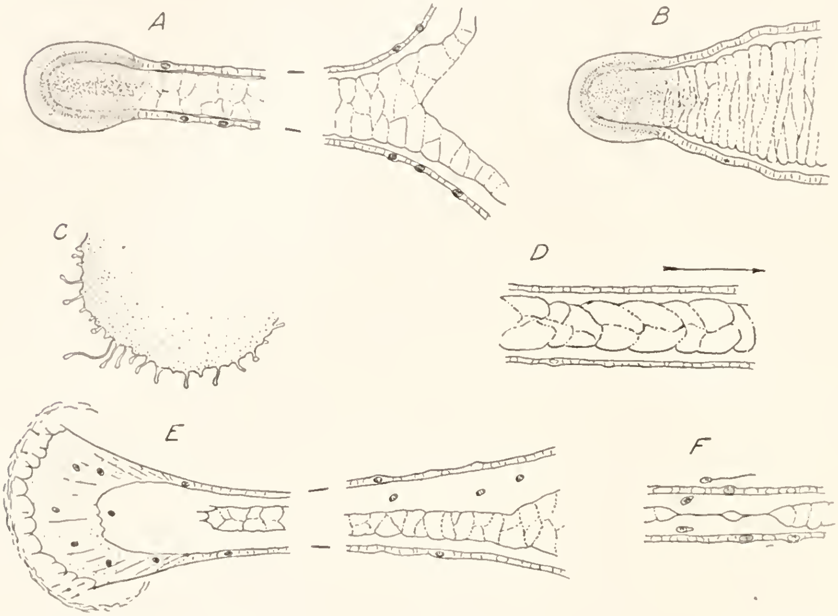

Fig. 4. Living pedal stolons as seen with the high power of the microscope (optical sections). *A*, a young stolon with specialized ectodermal tip and core of entoderm. *B*, the same contracted as a result of mechanical stimulation. *C*, enlarged detail showing margin of a tip undergoing attachment to the substrate. *D*, a stolon under tension following attachment. The core of entoderm is migrating back into the stalk of the polyp in the direction of the arrow. *E*, attached stolon under tension. The adherent tip is anchored to the bottom of the dish by means of fibrils. The core of entoderm is partly withdrawn. *F*, an old stretched stolon. The entoderm has become reduced to a thread with a few cells scattered along it. Cnidoblasts are shown in this and other figures.

of a rhizopod protozoan; except, of course, that the stolon is composed of many cells.

The above description applies when one stolon is formed at a time. When several are formed, each one cannot become a base (Fig. 2*A*). Some develop tension without being able to shorten. After a day or so in this case also the entodermal core is withdrawn; and still later the ectoderm pulls away from its attachment and is withdrawn.

Rôle of the Germ Layers in Locomotion

Observations under the high power of the microscope make it clear that the two germ layers of the polyp play diverse rôles in the processes of budding and locomotion. We shall not discuss their parts in the development of a hydranth at this time, except to note that the entoderm comes very close to the ectoderm in the region where a mouth is forming, and in the regions where tentacles are about to grow out. (*a*) The first visible evidence of a bud or stolon, similarly, is a conical projection of entoderm as seen through the overlying transparent ectoderm. The tip of the entoderm seems to push away the intervening mesogloea and to come into actual contact with the ectoderm. (*b*) As the stolon grows outward a rearrangement of the entoderm takes place (Fig. 4*A*). The entodermal cells of the wall of the stalk migrate actively into the forming process and rearrange themselves into a solid core. The ectoderm, meanwhile, undergoes little change in its appearance and in the arrangement of its cells, except that it grows thinner. (*c*) An exception to the above statement is the ectoderm of the tip of the stolon, which thickens and becomes glandular. It is worth noting again that it is in this region that the ectoderm is in most close contact with the underlying entoderm.

If an elongated stolon be mechanically stimulated, a contraction of the ectodermal sheath takes place (Fig. 4*B*). The tip does not contract, and the entodermal core appears to be only passively compressed. Its cells become disc-shaped, and give the appearance in lateral view of a pile of coins. Contraction of this sort, however, is only temporary; and in a short while (an hour or two) the stolon is again fully elongated.

(*d*) Contact of the tip of the stolon with the substrate stimulates the glandular cells to discharge a cementing substance in the form of fibrils (Fig. 4*C*). On coming into contact with the solid surface the ends of these fibrils adhere firmly and so serve to anchor the tip of the stolon. Hérouard (1911) has described and figured a process of the formation of "tonofibrilles" which differs in some details from the above statement. As this process is taking place the tip of the stolon spreads out and flattens down in close apposition to the surface. A thin perisarc, a sort of mucus, is secreted around this area of attachment (Fig. 4*E*). (*e*) Contraction of the stolon follows. This begins with a flow of the core of entoderm toward the body of the polyp (Fig. 4*D*). The flow is most rapid in the middle, with the result that the entodermal cells become convex in the direction of the polyp's stalk. As the entoderm is withdrawn, the space between the ectodermal sheath and the entodermal core becomes wider and wider (Fig. 4*E*). If a stolon should be torn from its mooring at this stage of contraction, a contraction of the ectoderm takes place, and the narrow entodermal core is thrown into a spiral coil within the ectoderm. (*f*) On reaching the stalk the entodermal cells rearrange themselves again into the entodermal layer of the body wall. This involves, of course, the development of a cavity between them.

(*g*) As the entodermal core is withdrawn the ectodermal sheath develops tension. If the polyp is free to move, it is pulled toward the new point of attachment, which thus becomes the new base. If, however, the polyp is not free to move by reason of other attachments, the entoderm becomes drawn out into a thin axial thread with a few entodermal cells scattered along it (Fig. 4*F*). After thus serving for a time as an anchoring line, the ectoderm finally pulls away from its attachment and is withdrawn into the side of the stalk.

Morphallaxis of the Stalk

From the descriptions just given, it will be seen that the stalk portion of a polyp is of remarkable plasticity. It continually changes its form and structure as one stolon after another is formed and resorbed. The process is not to be classed as growth in the usual sense, since it does not involve an increase in the size and number of cells. Rather, the cells change their shape and to some extent their specifications. First, for example, the upper stalk cells become stolon cells with the tendency to form a solid process. Then, after the stolon has attached, they become progressively transformed into stalk cells again. Plastic molding of this sort was termed by Morgan " morphallaxis."

Usually when a stolon attaches and contracts, the stalk becomes bent downward toward the new attachment (Fig. 3*B*). In recovering its upright position, material is withdrawn (especially entoderm) from the old base and stolon (Fig. 3*C* and *D*). Looked at grossly there is thus a flow of materials of the stalk from the side on which old stolons are being resorbed to the side on which new stolons are forming. This movement was demonstrated in an experiment in which spots of vital dye

(Nile blue sulphate) were placed upon the side of the upper stalk. (The polyp was laid on a piece of paper and a crystal of the dye was touched to the surface for a few seconds. The polyp was then returned to sea water and any adhering dye was washed off.) In some instances stolons grew out from the region of the spot, or from just above the spot. It was then observed that the spots elongated into the stolons. Later, when the stolons were resorbed, the marked areas again became compact spots, but upon the side of the stalk opposite to that upon which they were originally placed.

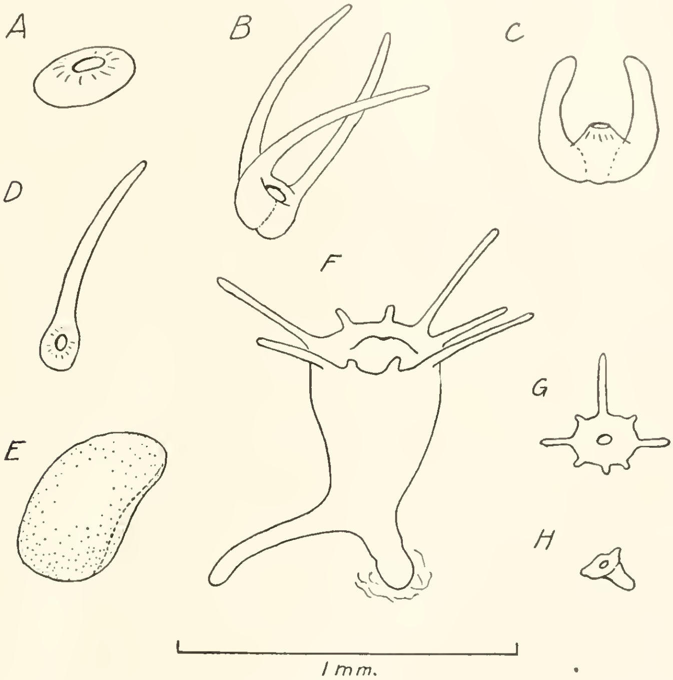

FIG. 5. Reconstitution from small pieces. *A* is a small piece taken from the oral disc (peristome). *B, C,* and *D* are tentacles with a small amount of adjacent material from around their base. *E* is a piece of entoderm which has rounded but not undergone regeneration. *F* is a comparable piece of ectoderm. It has formed a complete polyp. *G* and *H* have regenerated from very small fragments of ectoderm.

EXPERIMENTS

Reconstitution

Regeneration of Small Fragments.—Animals which possess the capacity for vegetative reproduction are as a rule also able to regenerate themselves from pieces. This is true of scyphozoan polyps. Indeed, no part of this animal is without some power of restitution, although the power differs from region to region both in degree and in kind.

Small pieces roughly one millimeter square isolated from different locations behave differently. (*a*) A piece cut from the oral disc (peristome), and including a part of the oral lip but no tentacles, will roll itself together and will regenerate one mouth, sometimes two mouths; but usually no tentacles nor any part of the body below the tentacles are formed (Fig. 5A). (*b*) A similar piece taken from the region of tentacles will close and regenerate lips and oral disc, sometimes additional tentacles, but usually no body (Fig. 5, *B* and *C*). If a tentacle has been cut off it will grow out again. Sometimes the stump of a tentacle will regenerate a bifurcated tentacle. (*c*) An isolated tentacle heals its cut end but otherwise undergoes little change. It may live for days, swimming about as though it were a ciliated worm. Occasionally a tentacle cut close to its base regenerates a small mouth (Fig. 5*D*). (*d*) A piece of the upper body removed from just below the circle of tentacles promptly regenerates an oral disc and tentacles. (*e*) From the upper body region downward to the attached base there is a gradual decrease in hydranth-forming tendency. The hydranths which are formed at lower levels are smaller, of fewer tentacles, and require a longer time for their regeneration. Considerable variability in hydranth-forming power exists, however, especially at the lower body and upper stalk levels. Some pieces form whole, well-proportioned polyps, and do so quite promptly. Other pieces from the same level form single large stolons, or even two stolons. No doubt this variability reflects the bud-forming or stolon-forming tendencies which were present in this region of the polyp at the time the pieces were isolated. (*f*) Pieces taken from the basal end of the polyp or near it usually round up into a ball and secrete a loose perisarc about themselves. (*g*) Isolated stolons commonly attach by their tips and then, after contracting, regenerate entire small polyps (Fig. 9*A–D*). The gradient of hydranth-forming tendency, which has just been described, is illustrated in the behavior of transverse segments as shown in Fig. 6. Segments *b* and *c*, which are from the upper body, have their hydranths fairly well reformed on the third day. Segments *d*, *e*, and *f*, which are from the lower body and upper stalk have small hydranths on the seventh day. Stolons are most advanced in segments *c*, *d*, and *e*. Segment *g* has rounded up and secreted perisarc. Later it may break from its perisarc and regenerate a very small polyp.

These observations show that invisible differentiation (segregation, chemo-differentiation, determination) is present in the polyp. No two parts behave in the same fashion when they are isolated. The invisible differentiation is not complete (stable or irreversible determination) except apparently in the oral lips and tentacles. Elsewhere large powers of regulation remain; or in other words, invisible differentiation is incomplete (labile determination), and redifferentiation is possible. Invisible differentiation reveals itself principally in formative tendencies; that is, in hydranth-forming tendencies which are strongest at the upper end of the polyp and decrease toward the lower end, and in base-forming tendencies which are strongest at the lower end. The power to form typical pedal stolons is greatest in pieces from the upper stalk area.

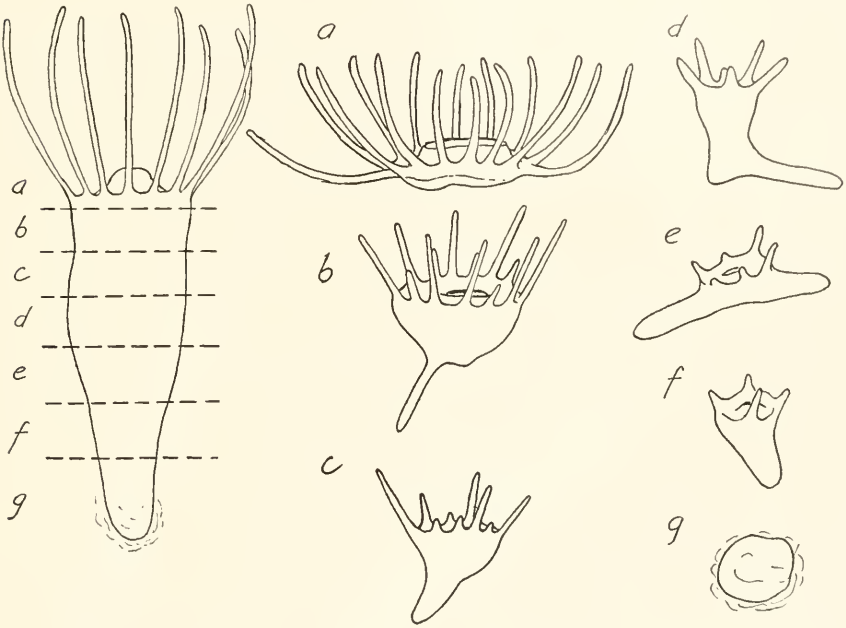

FIG. 6. Reconstitution from transverse segments. Note the decreasing hydranth-forming tendency from *a* to *g*; the pronounced stolon-forming tendency in *c* to *e*; the base-forming behavior in *g*. *a, b,* and *c* were drawn on the third day; *d,* on the fourth day; *e, f,* and *g* on the seventh day.

Polarity in Regeneration

Polarity in the scyphistomas is similar to the same phenomenon in *Hydra* and the hydroids. In general, transverse sections regenerate apico-basally; that is, with the hydranth formed from the upper cut surface (Fig. 6, *b* to *f*). Sometimes, however, sections cut from just below the circle of tentacles regenerate biapically; that is, with a hydranth from both the upper and the lower cut surfaces (Fig. 7*A, B,* and *C*). This is most likely to occur if the section be thin.

Longitudinal halves and quarters of polyps from which the hydranth has been removed regenerate asymmetric hydranths, which reveal both the inherent polarity of the piece and the orienting effect of wounding. If the piece be long the new hydranth is formed from the upper cut end; but the hydranth is oblique, facing inward as well as upward (Fig. 7, *D* and *E*). If, however, the piece be short, the new hydranth may be entirely symmetric and face directly inward (Fig. 7, *F* and *G*.) The oral lips and tentacles are in this case formed from the cut margins of the piece. Indeed, the wound may never close, as in cases in which the entoderm of the gastric ridge is disproportionately large (Fig. 7, *H*.)

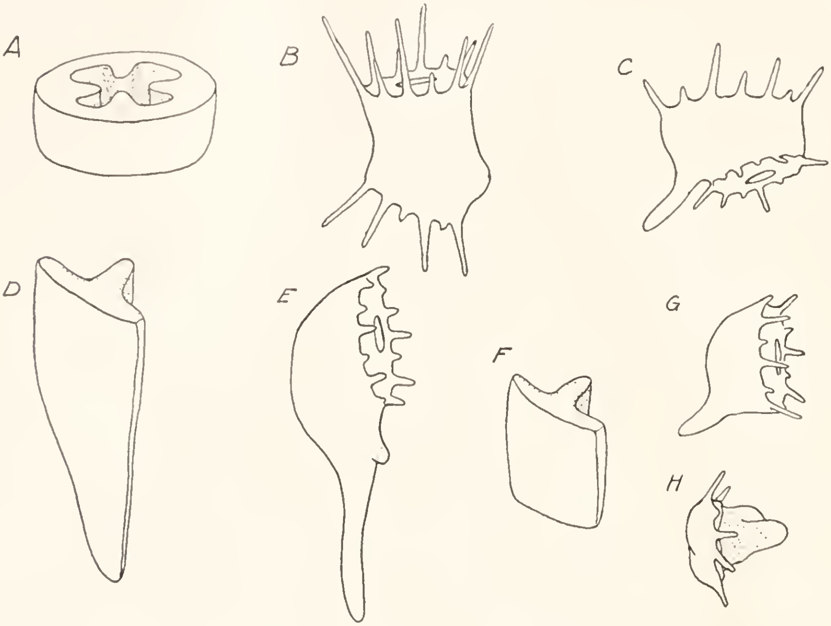

FIG. 7. Polarity in regeneration. *A, B,* and *C*. Thin transverse sections from the upper body region frequently regenerate biapically. *D* and *E*. A longitudinal quarter of a polyp (hydranth removed) regenerates obliquely, that is, with the hydranth facing upward and also inwards. *F* and *G*. A quarter of a body segment regenerates with the hydranth facing inwards, at right angles to the original polar axis. *H*. Similar to *G* except that the large entodermal gastric ridge prevented closure of the wound. Oral lips and tentacles have formed from the margin of the wound.

These facts suggest that polarity in scyphistomas is less a matter of orientation (for example, of molecules or of "intimate structure") than it is of gradation. The upper body portion is strongly hydranth-forming. Moreover, the hydranth-forming potential is not greatly different between the opposite surfaces of thin pieces taken from just below the tentacles. Were polarity primarily orientation, the proportion of biapical regenerates would not increase as sections are cut thinner. If the basis of polarity is orientation, it must be of a very labile nature, to account, for example, for the change in the direction of polarity which results from wounding.

Unlike the hydranths, the pedal stolons do not form at cut surfaces. In the case of biapical regenerates they form from the side of the piece. In monapical regenerates they form from uninjured body wall just to one side of the healed lower cut surface. In longitudinal pieces the stolons form from the non-wounded surface (see later). It is obvious that stolon formation obeys different laws with respect to polarity and with respect to the effects of wounding, from the laws of hydranth formation.

Regeneration from Ectoderm and Entoderm

It is fortunately possible to separate pieces of ectoderm and entoderm and to observe the behavior of these two germ layers separately in explants. It is then found that entodermal pieces round up into ciliated balls, which may remain alive and rotating for several days, but which do not regenerate (Fig. 5, *E*). Pieces of ectoderm, on the other hand, round up, and within a period of 7 to 11 days regenerate small, complete polyps (Fig. 5, *F, G,* and *H*). The evidence indicates that entoderm is irreversibly differentiated as entoderm, whereas ectoderm is labile and dependent for its differentiation. (The reversibility of the ectoderm is presumably due in whole or in part to the presence in ectoderm of so-called interstitial or restitutional cells.) Whether or not the entoderm is regionally differentiated has not been directly determined. There is, however, indirect evidence that it is.

When ectoderm is isolated alone it rounds up and within a few days regenerates fairly well balanced polyps, the number and size of the tentacles of which depend on the size of the piece (Fig. 5, *G* and *H*). The form of the regenerate has little relation to the region of the polyp from which the ectoderm is taken. (Ectodermal explants have not been made from the oral disc, tentacles, or base.) Pieces from the stalk region, however, are somewhat slower regenerating than pieces from the body. When ectoderm and entoderm are isolated together, the regenerates reveal very distinct regional differences, as has already been described. These observations indicate that regional differentiation exists primarily in the entoderm, and that the ectoderm is largely dependent upon the entoderm for its regional characters.

LOCOMOTION

Experiments on the Origination of Stolons

In the preceding section we noted that the pedal stolons of the scyphistomas typically form from the upper portions of the stalk, and that as they grow outward they tend to point upward away from the base. They also have a tendency to form on the side of the stalk away from the remnants of older stolons. These facts suggest that the base and the older stolons have an inhibiting influence upon the formation of new stolons.

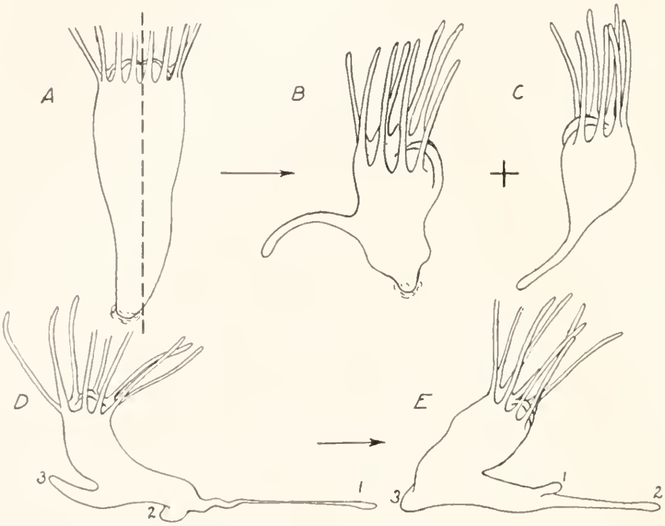

FIG. 8. Pedal stolons formed by half polyps. *A*. A polyp has been cut approximately into halves. *B*. The piece which retains the base forms a typical pedal stolon from the side opposite the cut. *C*. The piece which lacks the base forms a short stolon from near the lower end. *D*. A half polyp in locomotion toward the left. *E*. Later stage of the same polyp.

To test this assumption two experiments were performed:

(1) Twenty polyps were selected which showed neither buds nor stolons, and to which no injury had been done. A bit of the calcareous substrate adhering to the base was taken as evidence that the base was intact. From ten of these polyps the base was then cut away. Twenty-four hours later about equal numbers of the operated and the unoperated polyps (five and six respectively) had produced stolons. The rate of stolon formation is therefore not greatly, if at all, influenced by

the removal of the base. But the stolons of the two groups were characteristically different. In all but one instance, the operated animals produced stolons which grew downward from the lower end of the remaining stalk; while the control group produced long, typical pedal stolons which grew upward and outward from the side of the stalk. The experiment was repeated with similar results.

(2) Twenty polyps were again selected as before, but were cut longitudinally into halves, except that in each instance the cut passed just to one side of the base (Fig. 8, *A*). Forty-eight hours later essentially the same numbers of each group had produced stolons (15 of those with base; 17 of those without). In most cases those without a base had produced short, downward-growing stolons (Fig. 8, *C*); while all those with a base had formed upward and outward-growing stolons (Fig. 8, *B*). Thus again the presence of a base influenced the place of origin and the direction of growth of the stolons.

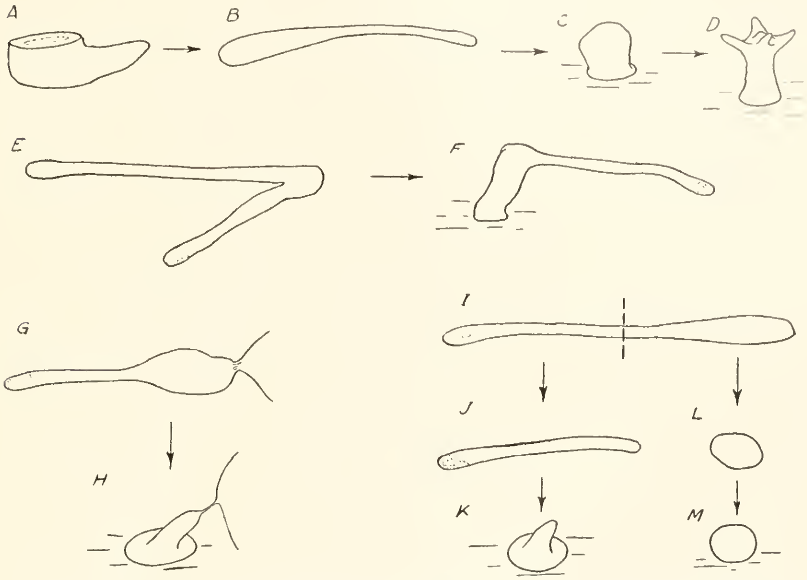

FIG. 9. Operations on pedal stolons. *A*. A section of the stalk including a beginning stolon. *B, C,* and *D*. The stolon elongates, attaches, and contracts, and in due time regenerates. *E*. An isolated stolon which has developed a branch stolon at its base. *F*. The original stolon has attached and contracted; but the branch stolon remains unattached and elongated. *G*. A pedal stolon was crushed near its proximal end and immediately contracted in the region of injury (temporary contraction). *H*. Later the tip attached and permanent contraction took place. *I*. An isolated stolon was cut into two pieces. *J* and *K*. The distal piece remained elongated until its tip attached, when it contracted. *L* and *M*. The proximal half immediately rounded into a ball which later adhered lightly to the bottom of the dish.

A further observation in connection with this latter experiment is important: The stolons always grew from the uninjured side of the half polyp. This was not only true of the first stolon, but of several succeeding stolons as well (Fig. 8, *D* and *E*); with the result that the partial polyps locomoted in one direction for a time; namely, in the direction away from the injured side.

These observations may be summarized by saying that (1) the rate at which stolons are formed is little if at all influenced by the presence or absence of a base, or by wounding; but (2) the position and direction of outgrowth of each new stolon is definitely affected. New stolons commonly grow from that portion of the stalk which is farthest from the base, and on the side opposite to the remains of older stolons. New stolons also grow from the side opposite a lateral wound.

Experiments on the Elongation of Stolons

A pedal stolon begins as a blunt cone, first of entoderm, then of entoderm and ectoderm as well. Within the space of a few hours it elongates until it has become a long, thin, tendril-like process with a solid core of entoderm. What processes are involved? We have already observed that elongation is a process of morphallaxis, in which the growing stolon draws to itself material from adjacent regions of the stalk. Experiment confirms this observation, and also indicates the primary rôle of the tip in the process of outgrowth.

(1) In several experiments beginning stolons were cut away along with a greater or less amount of the stalk (Fig. 9, *A*). In most cases the stolon continued to elongate, and in some instances (provided the base was not included) the entire fragment became transformed into a thin stolon of nearly uniform cross-section (Fig. 9, *B*). From this we conclude that the stolon is a "self-differentiating" system, physiologically independent of the polyp proper, and capable of exerting some measure of control over stalk material immediately surrounding the base of the stolon.

(2) In further experiments the tips of elongated stolons were crushed by pinching them with forceps. The stolons immediately contracted; and within the space of a few hours they usually had become completely withdrawn into the material of the polyp stalk. In some instances, however, when the injury was not too great, the tip was able to reorganize itself, or a new tip just proximal to the injured tip was formed. When this occurred the stolons re-elongated. Again, when the tip of a stolon was lightly stained with the vital dye, Nile blue sulphate, it remained extended; but when the staining was heavy, the stolon contracted. In several instances after such a contraction a new

tip developed just proximal to the stained region, and the stolon again elongated.

We conclude from these experiments that the outgrowth of a pedal stolon is a self-determined morphallactic process, independent of the polyp proper, but dependent upon the presence and activity of a free and uninjured tip.

Experiments on the Contraction of Pedal Stolons

The contraction (or retraction) of a stolon normally takes place immediately after its tip has attached to some solid object. If attachment fails to occur, the stolon may remain elongated for two or more days without contracting. The following experiments show that physiological continuity with an unattached tip is necessary if stolons are to remain uncontracted.

(1) It was regularly observed that when stolons are cut away from the polyp proper, they remain elongated until and unless the tips adhere to the substrate. The stimulation which results from injury at the proximal end of the stolon at most produces a slight, temporary contraction of the ectodermal sheath. When the tip adheres to the substrate, however, the isolated stolon immediately and permanently contracts into a ball (Fig. 9, *C*). Control is obviously from the tip. A week or so later the ball will have regenerated a tiny whole polyp with mouth and circle of tentacles (Fig. 9, *D*).

(2) In many instances isolated stolons developed a second tip, usually near their proximal end. When this occurred a branch stolon grew out (Fig. 9, *E*) under the controlling influence of the second tip. It was then noted that when one of the tips attached to the substrate, only that portion which was under the control of that tip contracted. The part under the control of the free tip remained elongated (Fig. 9, *F*).

(3) When isolated stolons are cut into two or three pieces, the distal pieces with their free tips invariably remain elongated until and unless attachment occurs (Fig. 9, *I, J,* and *K*). (The few exceptions were presumably due to injury to the tip.) The proximal and intermediate pieces, on the other hand, invariably round up into balls (Fig. 9, *I, L,* and *M*). Occasionally after rounding up, such a piece may develop a tip and then re-elongate; but in every case observed the time interval was long (at least twenty-four hours), and the phenomenon was clearly one of regeneration.

(4) When an elongated stolon is crushed by a pair of forceps at some point between the tip and its base, a wave of contraction spreads in both directions from the point of injury. The contraction which

moves toward the tip is temporary (it is apparently of ectoderm only), and indeed it never quite reaches the tip (Fig. 9, *G*). In a short time this distal portion is again elongated. The contraction which moves proximally, on the contrary, is permanent. (It presumably is a temporary ectodermal contraction followed by complete ectodermal and entodermal retraction.) A day later the proximal portion, in the case of isolated stolons, is rounded into a ball. In the case of stolons which have not been cut away from the polyp, the portion proximal to the cut becomes completely resorbed into the stalk, while the distal part may dangle as a loose appendage connected with the polyp by a thin filament of ectoderm only. In Fig. 9, *H* such a piece has attached to the substrate and contracted.

(5) Ligatures of fine thread were tied about elongated stolons, breaking the continuity of the entodermal core but not of the ectodermal sheath. It was then observed that the portions of the stolons proximal to the ligatures promptly contracted, and in many instances succeeded in drawing the distal portion through the ligature and into the wall of the polyp stalk. Any portion of the stolon which remained distal to the ligature, however, continued elongated.

These several experiments plainly indicate that the control within the stolon is a one-way control, that it is of the tip over more proximal levels, and that physiological continuity, especially of the entoderm, is essential if the stolon is to remain elongated. It is further noted that neither the stimulus of wounding, nor the effects of healing of the wound, have a permanent effect upon the stolon in producing contraction. Only the attachment of the tip, or injury to the tip, or the removal of the tip is effective.

It has been emphasized that the presence of a free tip is necessary if the stolon is to remain elongated. However, if the cut be clean and very close to the end of the stolon, so that a part of specialized ectoderm of the tip remains as a part of the stolon, the stolon may in some instances remain elongated and in due time attach and contract normally. It thus appears that the functional "tip" of the stolon is the terminal glandular region of the ectoderm.

Discussion

General Formative Principles

The polyps which we have just described come as near to being the plastic "candle flames" and "whirl pools" which Thomas Henry Huxley and others have discussed as any objects of animate nature, certainly as any objects among the Metazoa. However, they are no without a considerable degree of stability of structure. The upper body possesses a definite morphology which is relatively constant. The lower body and stalk, although plastic and changing, nevertheless perform according to certain general principles. Still, it cannot be said that these principles are always rigidly obeyed.

As a first principle we note that although the outgrowths which take place from the lower body and stalk possess one or the other or both of two potentialities; namely, the power to form hydranths and the power to form stolons; yet these two potentialities are not realized to the same extent and in the same way at the different levels of the polyp proper.

(*a*) The power to form a hydranth may be realized at any level below the polyp's hydranth. In the lower body and upper stalk, however, there is a sort of opposition between the parent hydranth and the bud hydranth, which leads to the bud promptly pinching itself away from the parent (fig-type of bud), and retaining its connection with the parent by only a thin strand of ectoderm. When the differentiation of a bud hydranth is delayed (stolonic buds), the pinching away from the parent is also delayed. In the lower stalk region no such opposition exists, and the buds which are formed here (hydra-type buds) long remain attached to the parent by a broad, fleshy union. Intermediate conditions are to be found at intermediate levels.

The experiments upon the regeneration of fragments show that the power to form hydranths is greatest in the material immediately below the circle of tentacles; yet buds do not normally form here. It is obvious that the hydranth of a polyp inhibits the formation of a bud and so of a second hydranth in the very region where the tendency to form hydranths in explants is strongest.

(*b*) The potentiality of stolon production is most pronounced in the upper portion of the stalk. It is here that the more typical stolons are produced; and it is here also that the buds (fig-type) are most certain to form stolons. In the lower stalk region there is an opposition or incompatibility between the polyp proper and the production of stolons, with the result that but few stolons are produced, and these are not typical stolons, but short processes which attach quickly. The buds of this region (hydra-type buds), moreover, are mostly without stolons.

Why does this incompatibility exist? A first hypothesis is that the base and the older stolons exert some sort of inhibitory influence upon the production of new stolons. Against this view, however, is the fact that the removal of the base has little if any influence on the rate at which new stolons form. A second possible hypothesis is that the entoderm in the region where new stolons form is in a sense "younger" entoderm, that is, it has not been involved in stolon-forming activity for a longer period of time.

In brief, the location and structure of the several types of buds and stolons illustrate a principle of opposition and toleration: opposition between parent hydranth and the bud hydranth, and between the base and the stolon; toleration, however, of hydranth for stolon, and of base for buds. Buds and stolons develop most frequently at intermediate levels because the material of these intermediate levels is less differentiated either hydranth-ward or base-ward than that of the upper and lower levels.

The Rôle of the Germ Layers

A second principle to be noted concerns the parts played by the two germ layers in budding and locomotion. The entoderm appears to play the independent rôle. Its cells rearrange themselves in characteristic fashion as the bud develops or as the stolon forms and elongates. The ectoderm appears to be more passive, responding perhaps to some sort of inductive stimulus of the entoderm, especially where the ectoderm is in close contact with the entoderm; or adapting itself to the form assumed by the entoderm. Except in the tentacles, at the oral lips, and at the tips of stolons little specialization of the structure of the ectoderm is to be observed.

There is experimental evidence that the entoderm is the principal seat of invisible differentiation. When small pieces of ectoderm from the body or stalk are isolated they constitute themselves into whole and fairly well-balanced polyps, and they do so quite irrespective of whether they are from upper or lower levels. Similar pieces of entoderm show a higher degree of differentiation by not regenerating. Pieces which are composed of both ectoderm and entoderm, however, regenerate, but show strong regional differences, both qualitative and quantitative.

Buds

Why do buds arise? There is no answer more satisfactory than that given by Child—that they arise as a result of physiological isolation when the region which becomes the bud escapes from the dominating and inhibiting activity of the parental hydranth. They do not arise near the upper end because the inhibitory activity of the parental hydranth is strongest here. They do not arise at the lower end because the hydranth-forming power is weakest here. They arise at an intermediate level. The pinching away of the bud from the parent's body accompanies the differentiation of the bud hydranth.

An important feature to note is that the pinching away of the bud is at first complete only so far as the entoderm is concerned. The bud may retain its connection with the parent by a strand of ectoderm for a considerable time. The morphogenetic correlations seem to take place within the entoderm.

Pedal Stolons

Origination.—The problem of the origin and behavior of pedal stolons is of special interest. The polyps produce pedal stolons (also buds) one after another more or less continuously. Isolated pieces sooner or later produce stolons. Why then do stolons originate? The answer is not " polarity," for the stolons usually form at intermediate levels, and in explants they sometimes arise very close to the hydranth. It seems as though something accumulates in less differentiated regions which leads to the production of stolons.

The earliest sign of a pedal stolon is a cone of entoderm on the side of the stalk, which pushes outwardly, displacing the intervening mesoglœa, and comes into close contact with the surface ectoderm. As soon as this occurs the ectoderm responds by becoming the " tip " of an outgrowing stolon. One is inclined to interpret the process as an " induction " comparable to that by which, in amphibian development, the chorda-mesoderm of the primary archenteric roof induces overlying ectoderm to become the neural plate. However, critical experiments upon this phase of stolon development have not yet been performed.

Elongation.—As soon as a specialized tip is present the stolon elongates; and it does so by drawing to itself material from the stalk. Having elongated, it continues in this state, so long as the tip is free, so long as it is uninjured, and so long as physiological continuity remains between the tip and the remaider of the stolon. Something which the tip does produces elongation. What may be the nature of this action?

A first suggestion is that the tip dominates in the physiological manner which Child has frequently described. The pedal stolon is then to be compared with a bud; its tip is the apex of the bud. According to Child a bud originates when some region on the side of the parent's body becomes so increased in its rate of physiological activity that it becomes " physiologically isolated " from the individuating forces of the remainder of the animal. The region then begins to grow away from the parent and to differentiate into a new individual. Child (1929) was able to produce such a bud experimentally in the hydroid *Corymorpha,* a form which normally never produces buds in nature, by merely wounding the side of the stalk of the polyp. The first suggestion, then, is that the outgrowth of a pedal stolon is an example of physiological isolation and comparable to budding. The tip of the stolon is physiologically dominant, the remainder of the stolon subordinate.

Several observations indicate against this hypothesis and emphasize the contrast rather than the similarity between the origin of buds and the origin of pedal stolons.

(1) The tip of a stolon never differentiates into the apex of a polyp; that is, into a hydranth. Indeed, the opposite occurs. When a stolon is cut away and attaches by its tip, it is very apt to regenerate a small hydranth; but in this case the apex of the hydranth is always formed at the proximal end of the stolon. In fig-type buds, similarly, the physiologically dominant apex of the bud becomes the oral region of a hydranth; but the stolon is an outgrowth from the side of the bud of a different physiological nature.

(2) A hydranth may be produced by the stimulation of wounding, but stimulation or wounding never produces stolons. Again, the opposite is the case. When a polyp is divided longitudinally, the stolons always form from the uninjured surface of the pieces. Moreover, stimulation or injury to the tip of an elongated stolon results in the immediate retraction of the stolon.

(3) The development of a hydranth seems to depend upon some general and quantitative property of the cells which produce it, such as a high rate of metabolism; but the development of a pedal stolon depends upon the presence of a definite specialized region which we have called the tip. So long as a part of this specialized tip is present, elongation continues; but if the specialized tip is removed, contraction takes place.

A second hypothesis to account for the control which the tip of a pedal stolon exerts over the rest of the stolon is that the tip is an organ of internal secretion and produces a hormone, which, diffusing into the entoderm, causes the latter to organize itself as the central core of a stolon. This hypothesis recalls the activity of the growth hormone of plants, auxin. It has been found that in plants the growing tips of most stems, petioles, flower stalks, and coleoptiles produce a substance which, diffusing from the tip into the regions immediately below, induce the cells of the latter to elongate in a longitudinal direction (see Went, 1935). May it not be that something comparable to this takes place in the pedal stolon? May we not suppose that the specialized glandular cells of the ectodermal "tip" of the stolon act as an organ of internal secretion and synthesize a "growth hormone" which diffusing into the entoderm causes the cells of the latter to arrange themselves into a solid core? May we suppose that this internal secretion of the hormone continues until the specialized cells come into contact with a solid surface (or are injured) and discharge their cementing substance to the outside? Internal secretion then ceases, the hormone supply is cut off, and the elongated stolon is retracted.

This hormonal hypothesis seems to cover the facts. However, before it can be more than a mere working hypothesis, it will be necessary to know more concerning the structure and interrelations of the germ layers at the tip of the stolon; and grafting experiments will have to be performed.

If we may provisionally accept this hormonal hypothesis, then it follows that within the polyp we have two different physiological types of control: (*a*) Within the hydranth we have dominance and subordination of the sort which Child has described. In relation to a gradient-field which is established, the localization and differentiation of the zones of the hydranth is accomplished. Dominance of this sort is not due to a specific influence emanating from the functional apex, but rather to the establishment of a labile configuration of forces with the apex as its center (see Gilchrist, 1937). (*b*) We have secondly, by hypothesis, hormonal control within the pedal stolon. The early differentiation of one small region as a specialized organ of internal secretion results in a definite arrangement of the cells in the surrounding area, and a stolon is produced.

Both controlling regions, the lips of the mouth and the tip of the pedal stolon may be termed apices or "centers of organization"; but the apex of the hydranth dominates because of its high rate of physiological activity and its influence on the configuration of forces in the gradient-field; while the apex of the stolon controls because of the activity of a specific product of its metabolic activity. The first type of control is quantitative and dynamic; the second qualitative and chemical. The first, to employ Child's (1921) terminology, is transmissive; the second is transportive. Moreover, the differentiation of the hydranth and the development of the stolon are different sorts of processes. The first is a relatively irreversible process in which cells become specialized as oral lips, peristome, or tentacles. The formation of a stolon, on the contrary, is entirely reversible. It is a temporary arrangement of the nature of a morphallaxis, which persists only so long as the hormonal control emanating from the tip persists.

Contraction.—We have described two different types of contraction, which we may designate as temporary and permanent contraction. Temporary contraction takes place when a stolon is mechanically stimulated, as by pricking it with a needle or pinching it with forceps. The

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wave of response is then seen to move in both directions from the point of injury, although the wave which moves distally may never reach the tip. Temporary contraction of this sort is never complete, and is apparently of the ectoderm only. Within the space of an hour or so the stolon is again fully elongated.

Permanent contraction, or better, retraction, is of an entirely different nature. It takes place normally when the tip of a stolon attaches to the substrate. Experimentally it may be produced by injuring or removing the tip, or indeed by merely breaking the physiological continuity by means of a ligature. The first evidence of such contraction is seen in the movement of the cells of the entodermal core of the stolon toward the stalk of the polyp. Permanent contraction is best thought of as a negative process; a sort of undoing of the factors which produce elongation. In terms of the hypothesis of a growth hormone, permanent contraction results from the disappearance of the hormone.

The ectoderm is active in physiological (that is, temporary) contraction. Certainly in the stolon, the entodermal core appears to be passively compressed or thrown into a spiral when the stolon is stimulated. The entoderm, on the other hand, appears to play the more active rôle in morphogenetic (permanent) contraction. It plays the primary part also in the plastic straightening into an upright position of the stalk of a polyp after it has been bent down as a result of stolon attachment. In these morphogenetic changes of form the ectoderm appears to be passive, or at least to do little else than contract and thus supply tension.

SUMMARY

The processes of budding and locomotion have been studied in a scyphozoan polyp, presumably the scyphistomas of *Aurelia*. It has been found (1) that the buds which develop near the hydranth of the polyp are quick to pinch away from the polyp (except for a connecting strand of ectoderm) and to migrate away by means of pedal stolons; whereas the buds which develop near the polyp's base may long remain attached. This indicates an opposition between the hydranth of the polyp and the bud.

(2) The stolons which develop at the upper end of the stalk of the polyp are quite commonly organs of locomotion as well as of attachment. They elongate, attach by their tips, contract, and so draw the polyp forward. The new stolon becomes the new base of the polyp, while the old base becomes drawn out and finally breaks from its attachment. The fact that new stolons form away from the base and away

from the older stolons indicates an opposition between base and young stolons.

(3) The entoderm apparently plays the primary rôle in the formation and differentiation of buds, and in the formation, contraction, and final resorption of pedal stolons. Entodermal cells of the stalk of the polyp rearrange themselves into a solid core as the stolon elongates; they actively migrate back into the stalk as the stolon contracts.

(4) Small pieces taken from any region of the polyp show some power of regeneration. In general, there is a decline in hydranth-forming potentiality from the upper to the lower end. Pieces from the hydranth (oral disc and circle of tentacles) are irreversibly determined to form parts of a hydranth. Pieces from the body or stalk may regenerate whole polyps, although the relative size of the regenerated hydranth decreases as the base is approached. Base-forming tendency is strongest at the lower end. The power to form pedal stolons is greatest in pieces from the upper stalk.

(5) Small pieces of ectoderm only may round up and regenerate whole polyps. Pieces of entoderm round up but do not regenerate. Pieces including both ectoderm and entoderm regenerate in a manner typical of the region from which they are taken. The entoderm is thus the seat of irreversible invisible differentiation (chemo-differentiation).

(6) Various operations were performed on pedal stolons and upon the polyps which produce them; such as fragmentations of the polyp, and injury, isolation, fragmentation, and ligation of the stolons. The results indicate (*a*) that new stolons commonly grow from portions of the polyp's stalk farthest from the base and opposite the remains of older stolons, also on the side opposite a lateral wound; (*b*) that the outgrowth of a stolon is a self-determined morphallactic process independent of the polyp proper but dependent upon the presence and activity of a free and uninjured "tip"; (*c*) that contraction immediately follows in a part of a stolon when the physiological continuity between the part and the tip is interrupted.

(7) It is suggested that the formation of the specialized ectodermal tip of a stolon is the result of an induction originating in underlying entoderm; and that the tip having thus originated acts as an organ of internal secretion in producing a "growth hormone." The hormone diffusing into the entoderm causes the entodermal cells to arrange themselves as the solid core of a stolon. The secretion of the hormone ceases when the specialized cells of the tip come into contact with the substrate and discharge externally.

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MITOSIS IN AMŒBA PROTEUS

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In 1935 the authors described the process of mitosis in *Amœba dubia.* This process was found to be so different from that described by other workers on large free-living amœbæ that it was considered desirable to investigate the phenomena attendant on fission in *Amœba proteus,* the other most common large free-living amœba. The first mitotic figure, a metaphase, to be described for *Amœba proteus* was that of Carter (1912). This author succeeded in obtaining and figuring this single stage of the process. In 1918, Doflein described a few mitotic stages. His description was incomplete for early and final stages of the process. Both Doflein and Levy (1924) figure and describe the division sphere of *Amœba proteus.* Levy's figures are in outline only and he includes no nuclear stages. Taylor (1923), evidently working with *Amœba proteus,* described phenomena which may be reasonably interpreted as pathological. Bělař (1926) describes from original but unpublished work several stages in mitosis. Although he considers these stages to be of mitosis in *Amœba proteus* we believe them to be of *Amœba dubia* or some closely related species. Chalkley and Daniel (1933) presented an account of mitosis in *Amœba proteus* and correlated very closely cytoplasmic configuration of division spheres with nuclear stages. These authors used only one method of fixation and one stain and all figures and descriptions were made solely from living animals or total mounts. In a later paper, Chalkley (1936) used additional stains including the Feulgen reaction. Our experience in working on mitosis in *Amœba dubia* has convinced us that a clearer understanding of the method of division in amœbæ can be had by supplementing such studies with the use of sectioned material. With this in view the present study of mitosis in *Amœba proteus* was begun.

Material and Methods

Two different strains of *Amœba proteus* Pallas (Leidy) (see Schaeffer, 1916) were used in this work. One strain was isolated from a culture collected from a pond in the vicinity of Woods Hole, Mass., in 1924 by the senior author. The second strain was obtained through the kindness of Mr. J. I. Phinney who originally collected the amœbæ from Jamaica Pond, Boston, Mass. Specimens of the latter have been cultured in this laboratory since March, 1936. The method of culture of both is essentially similar to that published by Dawson (1928).

The technique for isolating, fixing, staining, etc. was similar to that used in the work on *Amœba dubia* (1935).

Dividing amœbæ were obtained in profusion at any desired time by the following method. Thriving cultures were placed in a refrigerator at a temperature of 3° to 4° C. overnight and set out next morning at room temperature, approximately 27° to 29° C. Depending upon the room temperature, amœbæ were found dividing in the greatest number at periods varying from 2 to 4 hours after removal from the refrigerator. This method obviates much tiresome watching of cultures for dividing amœbæ.

The following fixatives were used: Schaudinn's, Schaudinn's with 5 per cent glacial acetic, Bouin's (Allen's B–15 modification), Flemming weak and Carnoy and Lebrun's fluid. For total mounts both Carnoy-Lebrun and weak Flemming were used. In sectioned material for finer detail Flemming proved best.

The following stains were used: Heidenhain's hematoxylin (Grübler) aqueous; picrocarmine aqueous; Delafield's hematoxylin; safranin, both aqueous and alcoholic; Flemming triple stain and alcoholic Heidenhain's hematoxylin. The ferric chloride method used by Chalkley and Daniel (1933) proved very unsatisfactory as compared with other stains and consequently was used but little in this work. Heidenhain's hematoxylin was used in both long and short methods. Differentiation was done with aqueous iron alum and in other cases with a saturated aqueous solution of picric acid and ammonia following the method recommended by Kidder (1934). Flemming triple stain was used with sectioned material in a few instances but in such cases only the safranin stained. Concentrated aqueous safranin was used regressively and gave a very brilliant red color. Alcoholic safranin (Lee) in anilin water produced very good results in sections showing dividing chromatin.

With the Feulgen nuclear reaction, contrary to our experience with *Amœba dubia* and also to results generally obtained with rhizopods, a positive reaction was obtained. We agree in this respect with Chalkley's (1936) findings. In some cases, counterstains such as eosin were used after Heidenhain's hematoxylin.

The best results were obtained using alcoholic Heidenhain's hematoxylin which was prepared in the following manner. A ripened stock solution (10 per cent in absolute alcohol) was used to make a 0.5 per cent solution in 70 per cent alcohol. As a mordant with this stain, 4 grams of iron alum in 100 cc. of 70 per cent alcohol were used. It was found that much better results could be obtained when the staining was done at a temperature of 40° C. for about 15 minutes, i.e., mordant for 15 minutes and stain for 15 minutes. For differentiation the mordant solution was diluted twice. Differentiation was accomplished under a binocular dissecting microscope or the low power of the compound microscope. After differentiation, slides were transferred to 50 per cent, 30 per cent alcohol and then washed thoroughly in running tap water for 30 minutes. Dobell (1914*b*) found that this procedure gave excellent results in similar work.

Nuclear Structure of Vegetative Amœba

Leidy (1879) describes the nucleus in the living animal as being " colorless, homogeneous, indistinctly and finely granular, or more coarsely, uniformly, and distinctly granular. I did not at any time distinguish a distinct membranous wall to the nucleus; and a distinct nucleolus, if present, escaped my notice." Calkins (1898) found in sectioned *Amœba proteus* that the nucleus consisted of a more deeply staining substance—" chromatin in the form of granules distributed throughout the nucleus; the other substance has the form of a disk lying in the center of the nucleus." According to Schaeffer (1916) the nucleus in the living *Amœba proteus* is " typically discoid, sides of the disk flat, slightly convex or slightly concave; sometimes dented or invaginated, especially in old individuals; conspicuous, except immediately after division; size of discoid nucleus, average diameter 46 μ and average thickness, 15 μ. Chromatin in several thousand masses arranged in one (?) layer under the nuclear membrane." In a later paper (1926) the same author classes the nuclei of *Amœba proteus* (*Chaos diffluens*) and *Amœba dubia* (*Polychaos dubia*) under the same general type and states that in this type the chromatin occurs in the living ' resting nucleus ' " in a layer of small grains of uniform size at a greater or less distance from the nuclear membrane. . . . In addition to these masses of chromatin there are found, after fixing and staining, other masses of stainable matter, usually irregularly placed, nearer the center of the nucleus, and in some species ' clouds ' or concentric rings of fine dust-like stainable particles, whose chromatin constitution has not yet been fully established." Doflein (1918) who, in our opinion, undoubtedly worked with *Amœba proteus* gives a description of the vegetative nucleus which generally conforms with those given by Calkins and Schaeffer. The Binnenkörper, he states, is not sharply marked off from the outer nuclear region although he figures it as a distinct body. In general its shape conforms to that of the entire nucleus. In Taylor's (1923) figures of the resting nucleus of *Amœba proteus* peripheral granules and karyosome are shown and her description is essentially similar to that of Doflein. Bělař (1926) states that the so-called Binnenkörper of *Amœba proteus* and related forms is a fixation artefact. Chalkley and Daniel (1933) working with *Amœba proteus* state that "the chromatin, . . . in the resting nucleus is distributed in granules or 'blocks' immediately beneath the nuclear membrane." Further description of the vegetative or resting nucleus is lacking although mention is made of the karyosome during the initial stages of mitosis. Later, Chalkley (1936) states "the granules of chromatin (sic) that lie in interkinesis just beneath the nuclear membrane play no part in the formation of the chromatin of the equatorial plate, . . . the chromatin granules of the plate arise entirely from the karyosome."

The nuclear membrane of the resting nucleus of *Amœba proteus* is clearly defined in both the living and the stained preparations. Immediately under the nuclear membrane is a layer of deeply staining granules as described by various other authors. The size of these granules varies from 1 to 2 μ in diameter. From the study of total mounts alone a very inadequate idea is had of the nature of the remaining part of the nucleus. In stained sections the central portion of the nucleus may be accurately studied. Such preparations (Figs. 19–26) show the central portion of the nucleus to be composed of a lightly staining fairly homogeneous mass of finely granular material. To ascertain whether or not chromatin was present in this part of the nucleus the Feulgen nuclear reaction was used. By this method (Figs. 28 and 29) both the peripheral granules and the central portion stained, the former being somewhat more intense in its reaction. According to Chalkley (1936) the peripheral granules "contain at best merely traces of nucleic acid." Similar results were obtained by the use of Heidenhain's hematoxylin after various kinds of fixation. Depending on the fixative used it was found, as Bělař (1926) has pointed out, that the presence of a clearly marked "karyosome" is evidence of a fixation artefact (Figs. 27, 28 and 29). Following the suggestion of Calkins (1933), the term endosome will be used in this work in reference to the central portion of the nucleus of *Amœba proteus*.

THE PROCESS OF FISSION AS SEEN IN THE LIVING AMŒBA

The formation of division spheres and the subsequent changes in the form of pseudopodia are very similar to those previously described by the writers for *Amœba dubia* (1935). See Plates I and II. Doflein (1918) figured the division sphere of *Amœba proteus* and Levy (1924) presented outline sketches showing the various changes in the division sphere during fission in this species. The most accurate drawings showing this process are those of Chalkley and Daniel (1933).

The "hyaline area" indicating the position of the nucleus during early division phases mentioned by Chalkley and Daniel could usually be seen under the binocular dissecting microscope. The present authors found, as in *Amœba dubia,* cytoplasmic currents and general internal activity occur, although from external view the animal seems to be completely inactive. Observations on the living division spheres under the highest magnification possible did not reveal the dividing nucleus. Various procedures were used in preparing the living division spheres for study. In none of the preparations, including those made by the agar method kindly suggested by Dr. Chalkley, could any of the early stages in nuclear division be seen. At the time of cytoplasmic division, nuclei could, however, be readily observed.

As shown in Plates I and II the cytoplasmic division may follow one of two types both of which also may occur in *Amœba dubia.* The first type of division is shown in Plate I. Here in Figs. 1–5 are shown slight progressive changes in a living division sphere prior to elongation. A slight elongation can be noticed in Fig. 5. In Fig. 6 projections indicate the beginning of formation of the daughter amœbæ. These projections grow, pushing out in opposite directions and the remaining portion of the division sphere becomes drawn out into a cylindrical form as in Fig. 7. The connecting cylindrical part gradually becomes thinner and the daughter cells have numerous short, blunt pseudopodia. As in *Amœba dubia,* cytoplasmic currents can be observed to flow, first in one direction and then in the other in this connecting strand. The pulling out process proceeds rapidly (Figs. 8–11) until, just before the connecting strand breaks, all motion of cytoplasm within it ceases. Each broken end snaps back toward its respective daughter amœba becoming broader and thicker as it is withdrawn. It is interesting to note that this type of division although most frequently met with in *Amœba dubia* occurred less frequently in *Amœba proteus* than the second type.

In the second type of cytoplasmic division no long connecting strand can ever be seen. The stages preceding either type of division are similar up to the point (Fig. 6) where indications of the forming daughter amœbæ become obvious. The two daughter amœbæ flow out very rapidly forming very large coarse pseudopodia (Figs. 12 and 13). The connection between them as shown in Fig. 14 is very short as compared with that in Fig. 11. From this point separation occurs within a few seconds leaving the daughter amœbæ lying close together.

In some instances it is difficult to detect the complete division until after fixation (Fig. 18).

Our results agree closely with those of Chalkley and Daniel (1933) in respect to the duration of the process of fission in *Amœba proteus*. In general it has been found that the correlations between pseudopodial width and stage of nuclear division as described by the above-mentioned writers are roughly correct. In our experience, however, wide divergences may exist between pseudopodial configuration and the stage of nuclear division. Division spheres which had already begun to elongate showed, upon fixation and staining, early stages of division. The pseudopodia are coarser in the later stages of fission. In this work, however, it has been found impossible to isolate any desired stage of nuclear division with the 97 per cent accuracy claimed by Chalkley and Daniel (1933).

THE PROPHASE

The resting nucleus of *Amœba proteus* which is entering on the prophase becomes considerably swollen so that when observed in surface view, although still discoid in shape, the slight, biconcave depressions have now pushed out. The nuclear membrane shows clearly, being somewhat thinner than in the vegetative condition. The peripheral granules still stain fairly strongly and have now decreased in number. They are much less uniform in size and more widely separated. At the same time there is forming, in the central portion of the nucleus (endosome) a mass of numerous small, deeply-staining, rod-like granules which are all definitely less than $0.5\,\mu$ in length (Figs. 43, 44, 45 and 60). These granules have a plate-like arrangement.

Spindle fibers are lacking at this stage. According to Chalkley and Daniel " the chromatin has left the membrane and is evidencing a tendency to aggregate in a zone . . . the chromatin appears completely

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EXPLANATION OF PLATE I

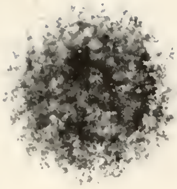
Photomicrographs. All figures were photographed using a Leitz compound microscope and Leitz apochromatic lenses. Number 3 objective and 15 × oculars were used in all cases. All figures were made from living amœbæ in process of division. Magnification approximately 90 diameters.

FIGS. 1–5. *Amœba proteus* Pallas (Leidy). Showing successive stages in division spheres prior to elongation. Compare with Figs. 17 and 18.

FIG. 6. Division sphere showing beginning formation of daughter amœbæ.

FIG. 7. Slightly later.

FIGS. 8–11. Showing cytoplasmic bridge connecting daughter amœbæ in successive stages. Type I division. See text.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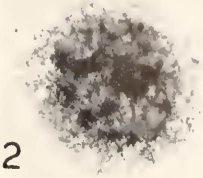
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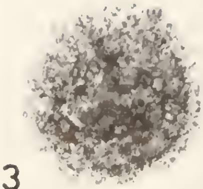
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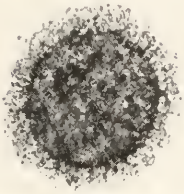
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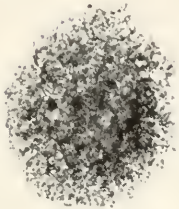
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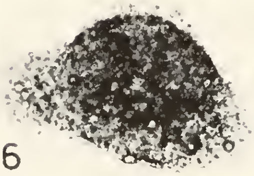
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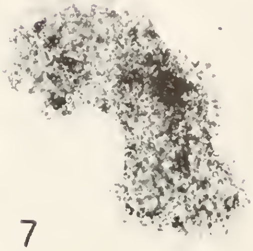
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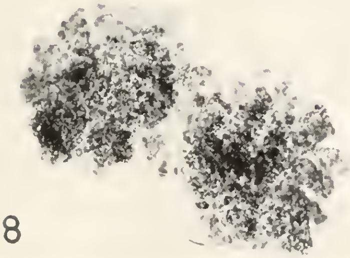
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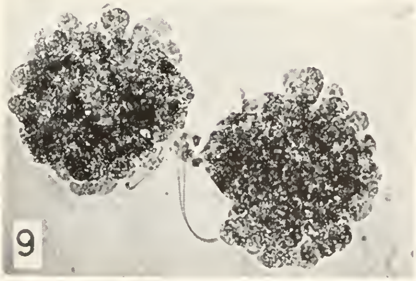
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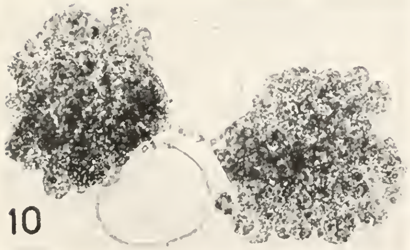
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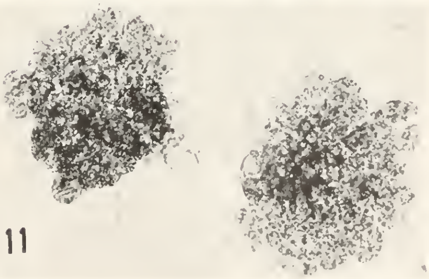
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PLATE I

withdrawn from the membrane . . . tending to form an irregular ring." Later (1936) Chalkley retracts this statement saying that the peripheral granules play no part in the formation of the equatorial plate. In our preparations no evidence can be found that the peripheral granules leave their position close to the membrane and migrate to a central portion of the nucleus. On the contrary, they gradually fade out of the picture and take no part in the formation of the plate. In preparations of this stage very definite evidence is present to show that the granules which are to form the plate are at no time arranged in a ring but are in the form of a disk.

The Metaphase

Beginning with the definitely formed metaphase plate as shown in Figs. 30–35, 46 and 61 the following condition is found. The nuclear membrane is still present. This is especially clear from our sections. Fibers are now seen extending from the plate to the nuclear membrane. At this stage, due to the shape of the nucleus and the delicate character of the membrane at the polar regions of the spindle, multipolar appearances have been observed. This effect is, we believe, due solely to fixation artefacts. The plate is composed of a very large number of individual, deeply-staining small chromatin granules. It is approximately circular in outline, the diameter being about 25 μ. The granules now are $\pm$ 0.3 μ in diameter and are quite uniform in size. In a few preparations, especially in sectioned material, larger achromatic granules may be observed. These lie outside of the plate among the spindle fibers. It is believed that these bodies are remnants of the former peripheral granules which have not yet disintegrated and that they take no part in the mitotic process (Figs. 35 and 46).

The dividing chromatin of the metaphase plate in *Amœba proteus*

EXPLANATION OF PLATE II

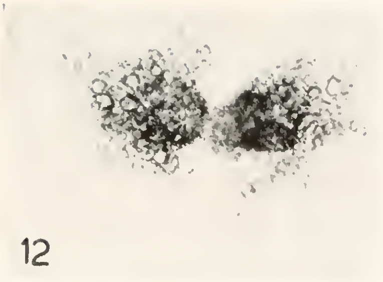
Photomicrographs. Figures 12–15 from living amœbæ in process of division. Magnification approximately 90 diameters.

Figs. 12–15. *Amœba proteus* Pallas (Leidy). Successive views of final stages in fission. Type II. Compare with Plate I.

Fig. 16. Total mount. Division sphere in prophase. Heidenhain's hematoxylin. × 70.

Fig. 17. Total mount. Division sphere in late anaphase. Safranin. × 400.

Fig. 18. Total mount. Cytoplasmic division, Type II. Note peripheral position of nuclei. Heidenhain's hematoxylin. × 400.



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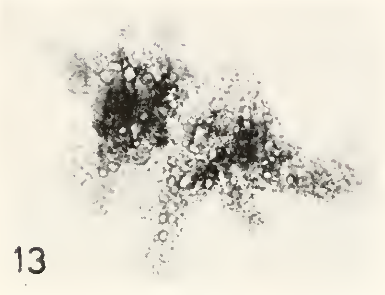

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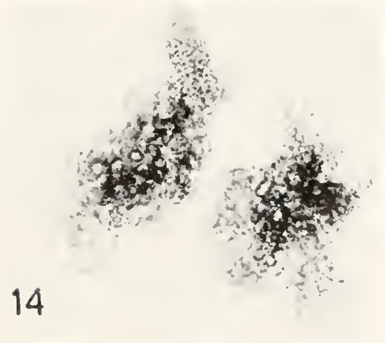

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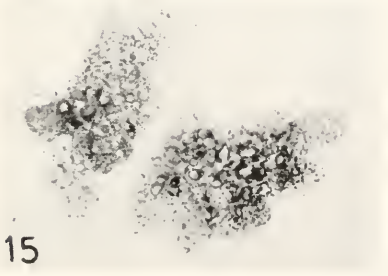

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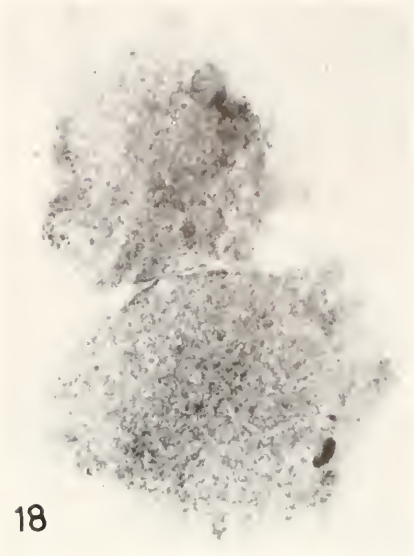

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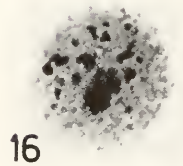

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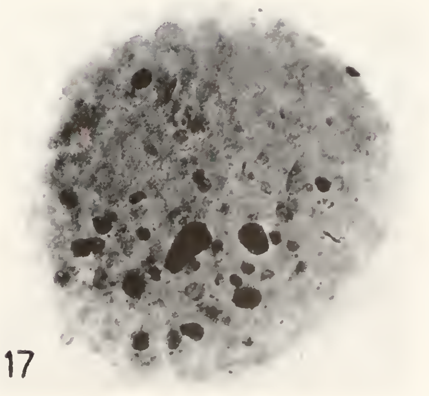

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PLATE II

stains heavily with all basic dyes used and gives a strongly positive Feulgen nuclear reaction. This is in sharp contrast with the condition in *Amœba dubia* in which no sharply defined metaphase plate was observed.

The Anaphase

In the early anaphase the former metaphase plate has divided to form two distinct plates (Figs. 36, 37, 47, 48 and 62). The nuclear membrane, although delicate, is still present and may be traced completely around the figure, especially in sectioned material (Figs. 36 and 62). The spindle fibers extending between the two separating plates can be clearly seen at this stage. They are extremely numerous. The polar fibers now definitely terminate at a point, giving a bipolar spindle. The chromatin granules comprising the plates are similar in size and staining capacity to those of the preceding stage. The larger achromatic granules now occur less frequently but a few may be observed in sectioned material (Figs. 36, 37, 47 and 48).

As the anaphase progresses the daughter plates become more widely separated (Figs. 38, 39, 40, 49 and 50). The nuclear membrane is still present. It is now very delicate and cannot be seen in all total mounts. In sections, however, its presence may still be detected. In

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EXPLANATION OF PLATE III

Photomicrographs. All magnifications × 1,200. All figures from sectioned amœbæ. Sections 6 μ thick. All fixed in Flemming except Figs. 27–29 which were fixed in Schaudinn's. All stained with Heidenhain's hematoxylin except Figs. 28 and 29 (Feulgen) and 30–34 (safranin).

Figs. 19–21. Vegetative nucleus serially sectioned through surface. Note deeply stained peripheral granules and in Fig. 20 endosome showing throughout.

Figs. 22–26. Vegetative nucleus serially sectioned at right angles to long axis.

Fig. 27. Vegetative nucleus showing endosome as fixation artefact.

Figs. 28 and 29. Vegetative nucleus. Feulgen. In both the peripheral granules stain deeply. Fixation as in Fig. 27.

Figs. 30–34. Serial section of metaphase plate. Note spindle fibers and nuclear membrane. Compare with Fig. 46.

Fig. 35. Early metaphase plate. Note achromatic granules and nuclear membrane.

Figs. 36 and 37. Successive views, early anaphase. Note nuclear membrane, divided plate, bipolar spindle and achromatic granules. Compare with Figs 47 and 48.

Figs. 38–40. Serial sections through mid-anaphase. Note nuclear membrane especially at polar regions, spindle fibers and achromatic granules. Compare with Figs. 49 and 50.

Figs. 41 and 42. Telophase, side and surface views from same amœba. Note "parachute" in Fig. 41 and convex type of plate. No achromatic granules present. In Fig. 42 note nuclear membrane and fine granulation. Compare with Fig. 54.

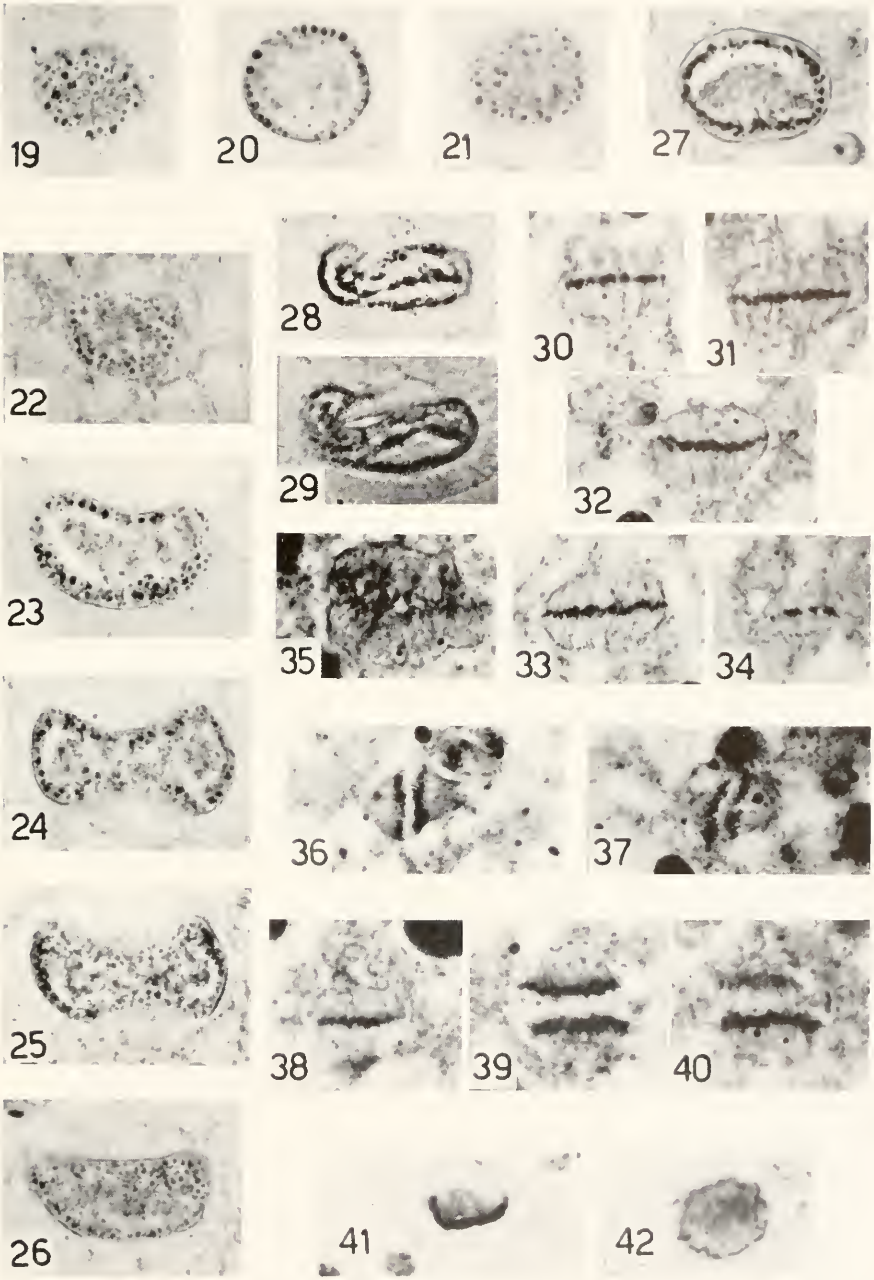
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PLATE III

mid-anaphase the spindle fibers are numerous and clear as are also the polar fibers. An excellent idea of the nature of the daughter plates in this stage may be had from a three-quarter view as shown in Figs. 51 and 63. The size and disposition of the chromatin granules in the plates are clearly shown.

The plates move farther apart and their planes still remain in most cases parallel. Each gradually becomes saucer-shaped with the convex surfaces facing each other (Fig. 52). The polar spindle fibers are still present but the fibers between the plates have practically disappeared. At this stage a few achromatic granules may still be seen in some preparations. In a similar stage in *Amœba dubia* the achromatic granules are relatively numerous.

The nuclear membrane so clearly visible in *Amœba dubia* at this stage becomes difficult to follow in *Amœba proteus*. In our preparations the nuclear membrane is present, surrounding the polar regions

EXPLANATION OF PLATE IV

Photomicrographs. All magnifications × 1,200. All figures from total mounts except Figs. 55–59 which are from sections, 6 μ thick. Figures 43–54 fixed in Carnoy-Lebrun. With the exception of Fig. 58 all remaining figures from amœbæ fixed in Flemming. All stained with Heidenhain's hematoxylin.

Figs. 43–45. Nuclei in prophase. Note peripheral granules and cloud-like forming plate. Compare with Fig. 60.

Fig. 46. Metaphase plate. Note achromatic granules. Compare with Figs. 30–35 and Fig. 61.

Figs. 47 and 48. Early anaphase. Two views of same figure at different focus. Note separating plates, spindle fibers and achromatic granules. Compare with Figs. 36, 37 and 62.

Figs. 49 and 50. Mid-anaphase. Two views of same figure at different focus. Note spindle fibers. Compare with Figs. 38–40.

Fig. 51. Mid-anaphase. Three-quarter view showing granulation of plates. Compare with Fig. 63.

Fig. 52. Late anaphase. Note curving of plates, polar fibers and achromatic granules. Compare with Fig. 17.

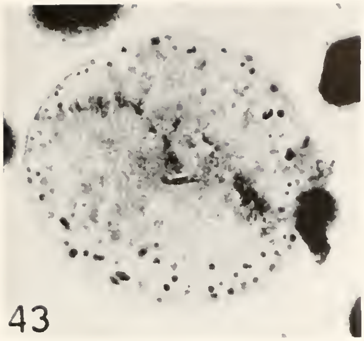
Fig. 53. Early telophase. Note greater curvature of plates and achromatic granules. Compare with Fig. 64.

Fig. 54. Later telophase showing one nucleus in side and one in surface view. Note character of granules and forming "parachute." Compare with slightly later condition in Figs. 41, 42 and 65.

Figs. 55–59. Showing reconstructing nuclei. Figure 55 one-half hour after division. Note small size of peripheral granules and vacuolated endosome. Figure 57. Two hours after division. Note typical nuclear shape. Peripheral granules increased in size. More granular.

Fig. 58. Three hours after division. Note increase in size of peripheral granules and those of the endosome. Compare with Fig. 68. Sectioned in surface view.

Fig. 59. Five hours after division. Note similarity to vegetative nucleus.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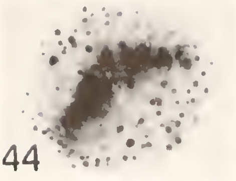
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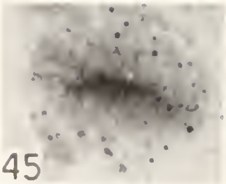
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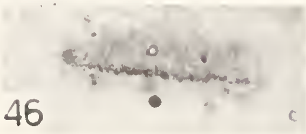
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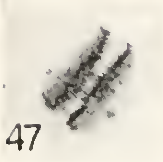
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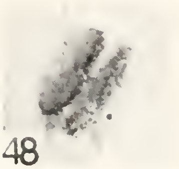
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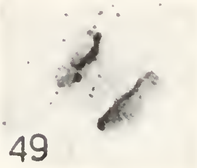
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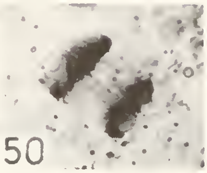
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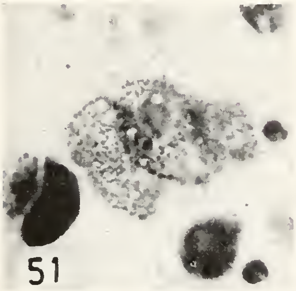
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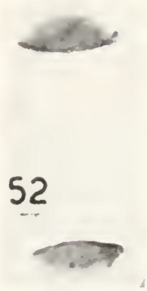
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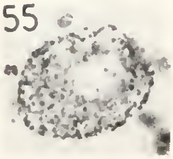
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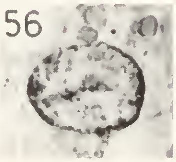
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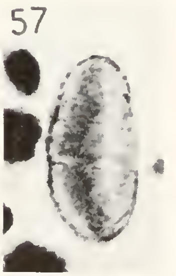
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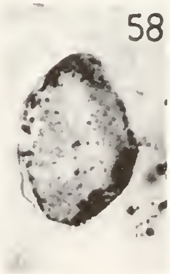
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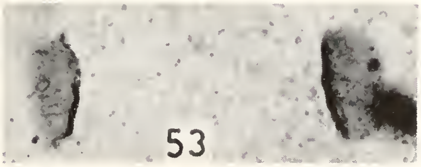
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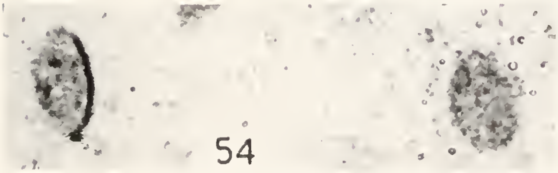
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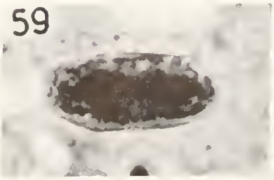
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PLATE IV

of the separating plates. It is difficult to make out with certainty on the inner surfaces of the plates. Some evidence of invagination or pinching off of the membrane in the equatorial zone of the spindle has been obtained. We believe that the membrane does not break down but divides in this manner just after mid-anaphase and thus comes to encircle each daughter nucleus.

The Telophase

The telophase stage is characterized by the further migration of the daughter plates (nuclei). At this time the division sphere has definitely elongated and the daughter plates (nuclei) are found near its poles. An early telophase is shown in Figs. 53 and 64. The daughter plates are now thicker, somewhat more convexly curved, and consequently have a shorter diameter. Measurements show the diameter now to be about 11 to 12 μ. The nuclear membrane appears much more distinct. The polar spindle fibers form a conical cap on the concave surface of the plate. No sign of interzonal fibers can be seen. At the same stage in the division of *Amœba dubia* such fibers are characteristically present. The disappearance of the achromatic granules is practically complete at this time.

Later stages of the telophase are associated with cytoplasmic division of the animal. Typical later telophases are shown in Figs. 18, 41, 42, 54 and 65. The nucleus may now best be described by calling it a "parachute." The polar spindle fibers represent the shrouds of the parachute and the plate, the dome. Such structure is most apparent in sections one of which is shown in Fig. 42. In surface view (Figs. 42 and 54) one can observe that the granules originating from the plate

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EXPLANATION OF PLATE V

All drawings made with camera lucida. × 1,700.

Fig. 60. Same as Fig. 43. Nucleus in prophase showing peripheral granules clearly defined and forming plate.

Fig. 61. Similar to Fig. 33. Median section through metaphase nucleus showing dividing plates, spindle fibers and nuclear membrane.

Fig. 62. Same as Fig. 36. Early anaphase showing divided plates, bipolar spindle, spindle fibers and nuclear membrane.

Fig. 63. Same as Fig. 51. Mid-anaphase, three-quarter view.

Fig. 64. Similar to Fig. 53. Showing early telophase condition. Note characteristic saucer shape of plate and achromatic granules.

Fig. 65. Late telophase nucleus. "Parachute" stage.

Fig. 66. Reconstructing nucleus ten minutes after division. Note uniform distribution of granules and delicate nuclear membrane.

Fig. 67. Reconstructing nucleus one and a half hours after division.

Fig. 68. Reconstructing nucleus three hours after division. Note presence of peripheral granules.

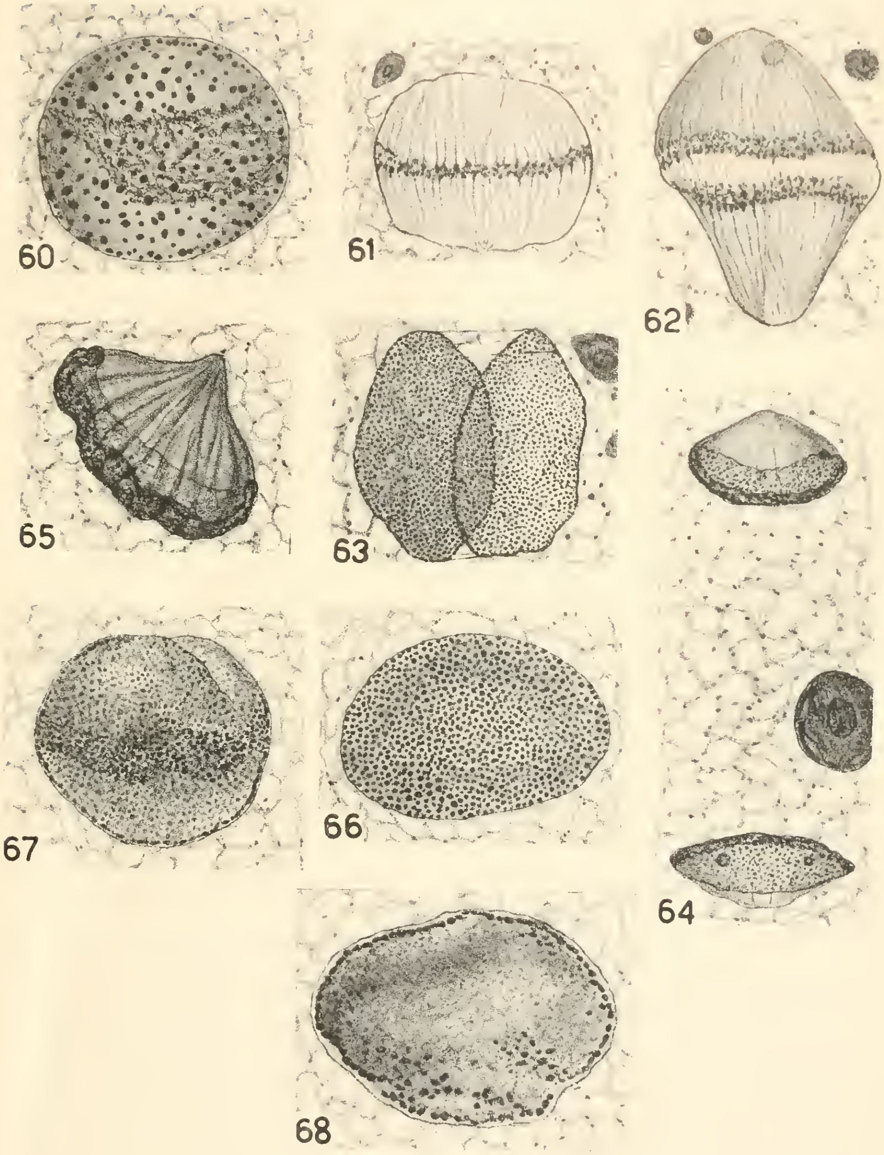
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PLATE V

are extremely fine and numerous. They do not stain so intensely. The diameter of the granules is unchanged. The diameter of the nucleus in surface view varies from 12 to 15 μ whereas the measurement of the short axis of the nucleus is approximately 4 to 5 μ At this stage but one type of staining granule is present and the nucleus is obviously much smaller than in the adult vegetative condition.

Reconstructing Nucleus

Although at the time of cytoplasmic division the nucleus has a definite membrane, it can by no means be considered completely reconstituted as Chalkley and Daniel (1933) state. We have studied the reconstituting nucleus both in total mounts and in sections from the time of division to over 5 hours thereafter. At the moment of division there is no evidence of the characteristic biconcave, discoidal shape (Fig. 18) but it resembles a thin saucer. Ten minutes after division it becomes slightly biconcave and measures approximately 20 μ in the long diameter and 6 μ in the short diameter. The former plate still occupies its central position in the nucleus. In surface view it is seen to be composed of numerous, extremely small (less than 0.3 μ in diameter) granules which stain less intensely than those of the telophase. During the interval between cytoplasmic separation and ten minutes after division, peripheral granules first make their appearance. These, in the beginning, are indistinguishable in size and staining capacity from the granules of the plate.

From 10 to 30 minutes after division the nucleus becomes slightly larger being approximately 24 μ in the long axis at 30 minutes. The peripheral granules also increase slightly in size now measuring from 0.5 μ to 0.7 μ in diameter. They appear somewhat irregularly placed and seem to have fibrillar connections. The central region which marks the former position of the plate becomes slightly granular, vacuolated and stains more faintly (Figs. 55 and 66).

At the end of one hour after division the nucleus is approaching normal vegetative size, measuring through the long axis approximately 30 μ and through the short axis 13 μ. The final definitive shape of the vegetative nucleus has been nearly if not entirely assumed at this time (Figs. 56 and 67). The peripheral granules are slightly larger measuring about 8 μ in diameter. The central portion of the nucleus, the endosome, is now vacuolated, has a reticular appearance and stains more heavily. It is definitely granular and extends throughout the entire nucleus.

Other than a slight increase in size, little change can be noted in the two-hour reconstructing nucleus (Fig. 57). Three hours after division both the peripheral and some of the central granules have increased in size (Fig. 68). The picture is not essentially different four hours after division. The peripheral granules, however, show a perceptible increase in size, being 0.9 μ in diameter. The central mass (endosome) is definitely granular and extends throughout the nucleus. It stains evenly and fairly heavily in sectioned material.

About five hours after division (Fig. 59) at room temperature (in this case 30° C.) it is difficult to distinguish the reconstituting nucleus from the vegetative condition.

DISCUSSION

From the preceding account of mitosis in *Amœba proteus* it is obvious that the process differs markedly from that in *Amœba dubia.* It is also to a certain extent at variance with the accounts given by other workers with the same species. Descriptions of the vegetative nucleus of *Amœba proteus* by former workers agree in general that a nuclear membrane is present and that a layer of peripheral granules underlies this membrane. In regard to the structure and function of the central portion of the nucleus or endosome considerable diversity of opinion exists. Thus Taylor (1923) describes an endosomal body which is clearly marked off from the outer region of the nucleus. Doflein (1918), although he does not specifically state a similar condition, has figured it as such. Our observations have led us to agree with Bělař (1926) that such a sharply marked-off endosome is evidence of faulty fixation and our study of the structure of the vegetative nucleus of *Amœba proteus* convinces us that the account given by Calkins (1898) is essentially correct. We are unable to confirm Schaeffer's statement (1926) that the vegetative nuclei of *Amœba proteus* and *Amœba dubia* belong in the same category. In *Amœba dubia* no endosome as such could be seen but the staining granules were evenly distributed throughout the nucleus. We believe that the uniformly distributed mass of relatively large granules in the *Amœba dubia* nucleus are homologous to the peripheral granules of the *Amœba proteus* nucleus. Both disappear during mitosis and both are reformed "de novo" from the dividing chromatin.

Doflein (1918), Bělař (1926) and Chalkley and Daniel (1933) all hold that the peripheral granules are the dividing chromatin and that the endosome supplies no chromatic elements. It is noteworthy that Chalkley in his recent brief account (1936) completely reverses his opinion in this regard and states that "these granules give rise to, or contribute to, the formation of the spindle fibers and the pole caps." Our work shows that the peripheral granules begin to disintegrate during the prophase and do not migrate to the central portion of the nucleus where the metaphase plate is forming. The metaphase plate originates from the endosome.

The multipolar metaphase figures shown by Carter (1912) and Doflein (1918) are, as can readily be seen from our photographs and figures, solely due to artefacts of fixation. It is noteworthy that such figures are shown from sections. We have found that the so-called "multipolarity" is accentuated in sections through the outer portion.

Chalkley and Daniel [1] (1933) claim that the nuclear membrane disappears at metaphase. Although Carter (1912) figures a nuclear membrane surrounding the metaphase figure, Doflein (1918) questions this and states that the nuclear membrane is not present at this stage. On the contrary, we have found the nuclear membrane clearly defined as late as mid-anaphase and also present in the early telophase. Indications of its presence in the intervening short period (at most five minutes) may also be seen. We therefore believe that the nuclear membrane does not break down during mitosis in *Amœba proteus.*

Our observations on the reconstructing nucleus in *Amœba proteus* have led to a better understanding of the origin of the peripheral granules. These begin to reappear just after cytoplasmic division and gradually grow in size with the growth of the nucleus. The genesis of these granules is decidedly similar in *Amœba dubia* and we are forced inescapably to the conclusion that they originate from the plate material, i.e., they are endosomal in origin.

Binary fission in *Amœba proteus* and *Amœba dubia* represents, we believe, the method of reproduction. The conception of Doflein that multiple division is a regular occurrence in *Amœba proteus* is, in our opinion, erroneus. The critical review by Johnson (1930) concerning diverse theories of reproduction in the large free-living species of *Amœba* and the recent work of Halsey (1936) serve to confirm this belief.

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[1] In a more complete paper appearing in December, 1936, shortly before this paper went to press, Chalkley stresses again the absence of the nuclear membrane during metaphase and anaphase in the division of *Amœba proteus.* Evidence is presented by us in this paper to show that the nuclear membrane is present during metaphase and early anaphase. We are in accord with Chalkley in finding that the dividing chromatin in *Amœba proteus* originates in the karyosome (endosome), but it is difficult to follow his reasoning when he states that the polar caps, the spindle fibers and possibly the "new nuclear membrane" are formed in part from the peripheral granules. A study of his paper fails to reveal evidence for such a belief. It does seem entirely probable, however, that the so-called peripheral chromatin is derived from the karyosome. The fact that it gives the Feulgen reaction for a short period during reconstruction while it is migrating from the karyosome to the periphery would clearly indicate that such is the case.

SUMMARY

1. In *Amœba proteus* the vegetative nucleus is a discoid, biconcave structure with a nuclear membrane, a layer of peripheral granules and a central endosomal mass.

2. During prophase the peripheral granules begin to disintegrate and the dividing chromatin originates from the endosome.

3. In metaphase the plate is fully formed and consists of numerous, small, deeply-staining chromatin granules. Spindle fibers, both interzonal and polar, are first apparent at this time. The interzonal fibers disappear at late anaphase. The polar fibers persist throughout the process.

4. The granules of the metaphase plate divide to form the two plates of the anaphase which continue to separate, becoming condensed and curved until the telophase stage.

5. The nuclear membrane is clearly present up to mid-anaphase and again at telophase. There is evidence that it persists throughout the entire process of mitosis.

6. The nucleus is fully reconstituted about five hours after cytoplasmic division. The peripheral granules are formed from the plate mass and appear a few minutes after division.

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REPRODUCTIVE CYCLES AND SUPERFETATION IN PŒCILIÏD FISHES

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INTRODUCTION

Reproductive cycles in the pœciliïd fishes are of unusual interest for three reasons: (1) this family of fresh-water fishes arose in the tropics and for the most part has remained under tropical and sub-tropical conditions where the great seasonal variations in light and temperature found in temperate and arctic zones do not exist; (2) all the species in the family are ovoviviparous. The embryos have fairly large yolk sacs and are retained in the ovarian follicles after fertilization until birth, necessitating a respiratory exchange between parent and embryos. The ovary consequently serves the double function of providing the female gametes and of providing a site and the proper conditions for retaining growing embryos; (3) superfetation occurs in some of the genera. In some instances two broods of embryos of different ages are present in the ovaries and in the most extreme case there may be at one time as many as six small separate broods.

Pœciliïd fishes have been reared in aquaria for years and some of the general facts concerning size of broods, intervals between broods, correlation between size of brood and female parent, variation of brood size and intervals between broods at the different seasons of the year, are well known. Bits of reliable information concerning reproduction in a number of genera and species are found in most of the popular books on aquarium fishes. One species in particular, *Gambusia affinis,* has been introduced into new areas in the tropics as a mosquito eradicator and its life history and reproduction have been well studied. Such studies have given relatively little attention, however, to the developing ovocytes within the ovary and the relation between these groups of ovocytes and the broods.

In order to study reproductive cycles effectively it has been found necessary to know the stages of development of the embryos and of the ovocytes within the ovary and to correlate this information with the records of the reproductive cycles. Furthermore, it is necessary to have as much of this information as possible from single females. Consequently, as far as possible, it has been the procedure to secure the brood-bearing record of an individual and then kill the individual at a definite period after the bearing of a brood in order to examine the embryos and developing ovocytes in the ovary. By following this practice throughout the year data have been secured upon the following relations: (1) the total number of broods produced within a year; (2) the relative time interval between broods; (3) the relative size of broods; (4) the exact time of fertilization of a group of eggs as related to the birth of a previous brood and as related to younger ovocytes; (5) the period of time over which fertilization takes place; (6) the variation in stage of development within a single brood; (7) whether or not fertilization occurs so as to form a new brood before the former brood is voided (superfetation); (8) the relation of the interval between broods and of the size of broods to environmental factors.

Any large collection of gravid females made in the field furnishes material for a study of the stages in the ovocytes as related to the age of the embryos being retained in the ovary. Superfetation if it exists can also be studied in this type of material.

In the present study the following species have been studied by the method of maintaining laboratory cultures and dating and dissecting the gravid females: *Gambusia affinis, Gambusia holbrookii, Gambusia panuco, Mollienisia sphenops, Mollienisia latipinna, Xiphophorus hellerii, Xiphophorus strigatus, Pseudoxiphophorus bimaculatus, Platypœcilus maculatus, Platypœcilus variatus, Lebistes reticulatus, Limia vittata, Heterandria formosa, Micropœcilia picta, Micropœcilia paræ, Pœcilistes pleurospilus* and *Quintana atrizona*. The following have been studied by the use of large numbers of living specimens in the field: *Xiphophorus hellerii, Xiphophorus strigatus* and *Pseudoxiphophorus bimaculatus,* at Cordoba, Mexico (March, 1932); *Pœciliopsis infans* at Octolan, Mexico (April, 1932); *Mollienisia sphenops* and *Gambusia panuco* near Monterey, Mexico; *Mollienisia latipinna* and *Gambusia affinis* in Louisiana. Another field study was made of *Brachyrhaphus episcopi* and *Mollienisia sphenops* at Barro Colorado Island in the Panama Canal Zone during the summer of 1936.

The writer is indebted to Dr. Stillman Wright for a large collection of *Pœcilia vivipara* from Parahyba, Brazil.

Reproductive Cycles

Various species of pœciliïds have been observed for years in aquaria and the observation has been made many times in some species that once a female attains sexual maturity she produces broods at regular intervals of about thirty days. All cyclical character seems to have disappeared in the reproduction of the male, the production of sperm being practically continuous after sexual maturity is attained. In other species it has been noted that the regular interval between broods is approximately fifteen days, while in still another species the young are born with less regularity, in smaller numbers and with less interval between the broods. Various theories have been advanced to account for the different lengths of cycles.

Some few observations have been made of brood production under natural conditions where it has been noted that the brood interval is much the same as that observed in the aquarium-reared specimens. A marked retardation of reproduction occurs during the winter months and it is much more marked under natural conditions than in aquarium-reared specimens.

It has been noted also that mature specimens obtained in the field and introduced into the laboratory do not produce broods with the regularity of laboratory-reared specimens. The broods of the wild specimens tend to be produced at longer and more irregular intervals even after the females have been adapted to laboratory conditions for periods of fifteen months. The young of these same females, reared from the first in the laboratory, produce broods with more regularity. The true and complete picture of the reproductive cycle as it occurs under natural conditions can only be obtained from material living under natural conditions and for this purpose collections of considerable size are being made at monthly intervals of *Gambusia affinis* at Key West, Florida, and of *Brachyrhaphis episcopi* in the Panama Canal Zone. A study of the ovocytes and the embryos in the gravid ovaries will make it possible to indicate the events in the reproductive cycle for all seasons.

Reproduction without Superfetation

In some species the birth of a brood is accomplished before any younger group of ova has matured sufficiently to be fertilized. Consequently only one brood will be found in the ovary at one time and the embryos will be in about the same stage of development. However, the extent to which younger cells have developed at the time the embryos are evacuated varies greatly and the following account will begin with those species in which the next younger group of cells is quite small and relatively undifferentiated.

Gambusia Type.—In *Gambusia*, females produce broods at about thirty-day intervals during late spring and summer. When eggs have been fertilized and during the time in which the embryos are developing, the cells of the next wave are small. They remain small and latent until embryos have been voided and then grow and differentiate rapidly until ready for fertilization. In an ovary containing a brood of very young embryos the oldest and largest cells are about .25 mm. in diameter. Approximately two weeks later, when the embryos are about to be born, the cells are still small, being only .3 mm. in diameter. Two or three days after the birth of the embryos these same cells have increased to a diameter of .7 to 1.5 mm., almost the maximal size attained at fertilization. Fertilization occurs over a period of about five days, beginning about seven days after the voiding of the last brood of embryos. Due to the length of the period of fertilization the embryos of a single brood may be relatively far apart in their developmental stages. In all other species examined the fertilization period is short and the embryos show less range in stages of development. *Gambusia affinis, G. holbrookii* and *G. panuco* are all very similar in the peculiarities described above.

A number of studies have been made upon the reproductive cycles of *Gambusia affinis* and *Gambusia holbrookii* (Barney and Anson, 1921*a*; Hildebrand, 1917; Kuntz, 1913; Seal, 1911; Seale, 1917). The work was concerned principally with the number of broods produced by wild specimens each year and the external factors which affect fecundity. As a result of these studies it was learned that the interval between broods in *Gambusia affinis* was about thirty days during the spring months and as long as eighty-five days during the winter if the specimens were kept in the laboratory and supplied with adequate food and warmth. It was also learned that under natural conditions several broods were produced during spring and summer but that reproduction ceased altogether during the winter. Barney and Anson correlated their study of the brood-producing record with the finding of embryos of different stages of development in the ovary and concluded: (1) that a single annual reproductive cycle occurs in *Gambusia* in its natural habitat; (2) that fertilization of all the eggs for an annual cycle occurred at the same time; (3) that the small cells in the ovary did not develop to maturity during the year, and (4) that after fertilization and partial development the embryos did not proceed at the same rate, a sufficient number to form one brood reaching the birth stage, being evacuated and being followed at approximately monthly intervals by other batches of embryos until all eggs that had been fertilized were voided.

Kuntz (1913), on the other hand, assumed that "A considerable number of ova reach maturity at the same time. These, being fertilized, give rise to a brood of young. After the birth of this brood another lot of ova reach maturity and, being fertilized, give rise to a second brood. Thus, perhaps, all the ova required for the several broods which are born during a spring and summer may be present in the ovary at the beginning of the season."

The writer is inclined to agree with Kuntz that different groups of ova reach maturity at different times and that each group is fertilized separately, for two reasons: first, all the young are born at practically the same time and none remain in the ovary; second, within ten days after birth another lot of ova, approximately equal in number to the brood born previously, has developed and is ready for fertilization. It may be implied from the statement of Kuntz that he assumes *Gambusia* has an annual reproductive cycle in which all the ova for the annual cycle are elaborated at the same time. These interpretations concerning an annual cycle fail to take into account that the pœciliïds arose under tropical conditions and that they have short, often-repeated cycles each similar to one complete annual cycle in a fish of the temperate zone. Under the alternating conditions of the temperate zone these short cycles are repeated as often as environmental conditions permit within one favorable season and then become retarded or cease until favorable conditions are re-established.

The ovarian cycle in *Mollienisia latipinna* and *Mollienisia sphenops* resembles that of *Gambusia*. During the spring and summer broods are born approximately thirty days apart. Ova destined for a new brood reach a maximal diameter of about .7 mm. by the time the embryos of the earlier brood are ready to be voided—about twice the size of the cells in *Gambusia* at the same stage. However, they grow rapidly and reach a diameter of over 2 mm. by the time they are ready for fertilization. Apparently fertilization does not extend over so long a period in *Mollienisia* as in *Gambusia* and the embryos, just after fertilization, do not show the wide range in stage of development characteristic of *Gambusia*.

Pseudoxiphophorus bimaculatus has an ovarian cycle very similar to that of *Mollienisia latipinna* and *Mollienisia sphenops*. However, the interval between broods is longer, being thirty-five to forty days during May, June and July. In an ovary containing a brood ready for birth the next younger group of cells has reached a diameter of only .7 or .9 mm. in diameter. After the brood has been evacuated, however, the cells grow rapidly to a large size, about 2.4 mm., before fertilization.

Lebistes Type.—The rhythm of brood production in this type is very similar to that in the *Gambusia* type. During late spring and summer broods are produced approximately every thirty days. A record of brood production by ten females of *Lebistes reticulatus* is shown in Table I. The data are taken from unpublished records of Dildine and the writer. It will be noted that the most regular production of broods and the shortest interval between broods occur in the late spring and early summer months. In late fall and winter months the interval between broods is nearly as long as in the late spring when broods are produced from twenty-two to thirty days apart. The specimens were kept under fairly constant conditions as regards food, temperature and salt and gas content of the water. No artificial lighting was employed, however, and the exposure of the specimens to light was therefore controlled by the seasonal waxing and waning of daylight. The increase and decrease in brood production paralleled the increase and decrease in daylight. To what extent increased exposure to light may serve as a stimulating agent to reproduction, possibly through the medium of a gonad-stimulating hormone from the pituitary, remains to be worked out experimentally.

Table I

Showing dates of broods produced by ten specimens of *Lebistes reticulatus*. Numbers under months indicate day on which brood is produced. Intervals between broods are expressed in days.

Specimen Number	January	Interval	February	Interval	March	Interval	April	Interval	May	Interval	June	Interval	July	Interval	August	Interval	September	Interval	October	Interval	November	Interval	December	Interval
1											27	25	22	27	18	35	22			46	6			
2											30	22	22	27	18	35	22			44	6			
3							5	29	4	31	4													
4							30	26	26															
5							21	39	29	22	20	26	16	31	16									
6							1	33	4	27	1	34	4											
7									22	28	19	23	13											
8	18																						9	40
9		60	28			39	8	28	6	27	2												20	
10					30			32	2															

In *Xiphophorus* and *Platypœcilus* the brood-bearing record is very similar to that in *Lebistes* with a tendency to a slightly longer period between broods. The same increase in the interval between broods during the winter months has also been noted.

Conditions in the ovary during various phases of the reproductive cycle could be determined only by using the period in which a brood is voided as a first stage and then obtaining regular stages a few days apart up to the time for the voiding of the next brood. Such a series was obtained during the height of the reproductive season. The findings in a number of cases are included here to indicate conditions within the ovary. (1) A specimen of *Lebistes* was sacrificed immediately after it had produced a brood of nine young. No young were found in the ovary and it was evident that all of the brood had been born during a three or four-hour period. In the ovary were found six ova approximately 1.4 mm. in diameter, orange-yellow in color and somewhat transparent. Four additional eggs similar in structure and color but varying in diameter from 0.8 to 1.0 mm. apparently belonged to this group. Presumably all would have been fertilized to form the next brood. Another group of eight cells represented a still younger set which would have been fertilized still later. These younger cells could be distinguished from the older group of cells by their smaller size and difference in structure. The younger group contained cells white in color, opaque and less than 0.5 mm. in diameter with more variation in size than in the older group of cells. It should be emphasized that there were definite gaps in size between the older and the younger group of cells and also between this younger group and a still younger mass of cells in various stages of development. (2) A second specimen was sacrificed and the ovary examined eight days after a brood had been born. Fertilization had recently taken place in a group of ova about equal in number to the brood born eight days before. Some of the fertilized eggs had reached an early blastula stage, some were in early segmentation stages and two had apparently not yet been fertilized. The eggs in the group as a whole varied in diameter from 1.2 to 2.0 mm. One of the larger eggs had not yet been fertilized while several of the smaller ones of the group had been fertilized. Therefore, size, except within the limits indicated, is not an index of the fertilizability of an egg. A second group of cells, about equal in number to the group undergoing fertilization, was opaque and slightly yellow in color. These cells varied in diameter from 0.4 to 0.7 mm. in diameter. (3) The ovary of a specimen killed twenty-one days after the birth of its brood of six contained a new brood of seven, the individuals of which were 5 mm. long. Seven transparent yellow eggs, varying in diameter from .6 to .9 mm., represented the next oldest group. Two were slightly opaque, indicating that they were slightly younger than the others. Fifteen cells, the largest of which was about .25 mm. and the smallest less than .1 mm. in diameter, were white and opaque or had transparent centers and opaque white layers at the periphery.

The development of cells in the ovary up to the time that they become mature and ready for fertilization may be summarized as follows:

1. A large number of transparent cells less than 0.1 mm. in diameter are ready to be drawn upon for differentiation.

2. During the first month some of these cells begin to differentiate and develop a white opaque layer at the periphery. A number of the cells reach a diameter of about 0.5 mm. and become white and opaque throughout. At the same time an older group of eggs and also a developing brood is present in the ovary.

3. During the second month the oldest members of this group of developing cells grow rapidly, become orange-yellow and translucent and reach a diameter of from 0.8 to 1.4 mm. by the end of the month. The number of cells undergoing this development is approximately equal to the number of individuals in a brood.

4. During the third month these orange-yellow translucent eggs comprise the oldest group in the ovary, the embryos of the previous brood having been born. During the first ten days of the month they grow to a maximal size of 2 mm., some being as small as 1.4 mm. in diameter, and at the end of this ten-day period all are fertilized. Variations in the stages of the young embryos indicate that fertilization does not take place in all simultaneously. Fertilization of all members of the group is accomplished within two days. During the remaining twenty days of the month the embryos develop and at the conclusion of the month they are voided. In the meantime younger waves of cells are passing through stages of development already described.

A comparison between the *Lebistes* and the *Gambusia* types indicates: (1) that, at the time of the birth of a brood of embryos, the ovocytes of the next younger group is much larger and better differentiated in *Lebistes* than in *Gambusia;* (2) that the period of time elapsing between the birth of a group of embryos and the fertilization of the next group of ova is shorter in the *Lebistes* type than in the *Gambusia* type.

Data obtained in *Xiphophorus* and *Platypœcilus* were strikingly similar to those obtained in *Lebistes.* The adults of *Platypœcilus* and *Xiphophorus* are larger than those of *Lebistes* but ova of the same age are equal in size. However, in *Lebistes* fewer eggs come to maturity at one time and the broods contain fewer individuals. Other species which have cycles like that of *Lebistes* or intermediate between the *Lebistes* and the *Gambusia* type are *Pœcilia vivipara, Micropœcilia picta, Micropœcilia paræ, Brachyrhaphis episcopi* and *Limia vittata.* Others not yet studied will undoubtedly be added to the list.

Quintana atrizona Type.—In this type there is a very short interval between the birth of a brood and the fertilization of the ova of the following brood. In a female killed and examined twenty hours after the birth of a brood, the next group of cells in the ovary was found to be fully developed. These ova, approximately equal in number to the brood just born, were 1.8 to 2.2 mm. in diameter. Some had apparently been fertilized a few hours before. Of this group—nineteen in all—seven had reached an advanced segmentation stage, nine were in earlier segmentation stages and three had not yet been fertilized. It was apparent that fertilization had taken place in most of the ova immediately after the evacuation of the previous brood and that fertilization in the remaining three cells would have been complete within a few hours more. Differentiation and growth of the ova next in line for fertilization had reached a final stage by the time the embryos of the previous brood were born and the interval between the two events was reduced almost to the vanishing point.

TABLE II

Illustrating production of broods in *Pœcilistes pleurospilus*

Date	No. of young in brood	Interval in days between broods
November 20	2	
December 16	2	26
January 8	2	23
February 1	2	23
February 21	3	20
March 15	3	22
April 3	7	19
April 25	7	22
May 10	8	16
May 24	9	14
June 5	9	12

If the ova should develop still more rapidly and reach their final stage of differentiation and growth before the older brood of embryos was evacuated a condition would exist in which it would be possible for fertilization to occur before the older brood had been evacuated and for two broods to exist in the ovary at the same time. Such a condition of superfetation does occur in the next species of pœciliids to be considered.

Reproductive Cycles Involving Superfœtation

Pœcilistes pleurospilus and Pœciliopsis infans.—Specimens of *Pœcilistes pleurospilus* were observed from November to May in order to secure records of the broods produced. Single specimens were then sacrificed at different intervals after the production of a brood to determine the state of development of embryos and ova within the ovary. The record of broods produced is shown in Table II. The record shows that: (1) the interval between broods was longest in the winter months and that it was becoming shorter in the early spring months; (2) the broods were becoming larger as the spring advanced; (3) in general, also, the broods were smaller than those of pœciliïd fishes in which superfetation does not occur; (4) during the spring months the interval between broods is much shorter than in *Lebistes.*

Stoye (1935) finds the interval between broods in this species to be ten to twelve days. This is apparently the record of specimens reproducing in May or June.

A female of *Pœcilistes pleurospilus,* killed two days after the last brood of eight young had been born, contained two broods of unborn embryos in the ovary. The older brood consisted of nine embryos which were well developed but far from the stage in which they would be born. The eyes were well developed and pigmented, and pigment cells were present over the brain, down the mid-dorsal line and along the lateral line. All embryos were in approximately the same stage of development. The second brood contained nine very young embryos, with only a few somites developed. A group of twelve cells about .5 mm. in diameter, together with many smaller undifferentiated cells, were also present in the ovary. Another female, sacrificed twelve days after she had produced a brood, contained an ovary in which two broods were well separated in age and also a group of developing ova nearly 2 mm. in diameter.

An abundance of preserved material of *Pœciliopsis infans* was available and forty-two ovaries were dissected. In every ovary two broods of embryos were found, together with a group of cells approximately equal in number to the embryos in a brood, well in advance of the development of a mass of smaller undifferentiated cells. Embryos of a single brood varied somewhat more in their degree of development than was the case in *Pœcilistes pleurospilus* but the least advanced member of the older brood was always separated by a considerable gap from the most advanced member of the younger brood.

The condition found by Henn (1916) in *Pseudopœcilia fria* and *Diphyacantha chacoënsis* indicates that the ovarian cycle of these forms is probably like that of *Pœciliopsis infans* and *Pœcilistes pleurospilus.* Henn dissected five ovaries and found in each a brood of large embryos, a second brood of small embryos with about the same number of individuals as in the first group and a small number of developing ova.

In the first two of these species, at least, the sequence of events for development of ova and embryos would be as follows: (1) a group of ova grow and differentiate until they reach a diameter of about 2 mm. At this point an older brood of embryos, *A*, is at the point of being born and a much younger brood, *B*, is also present in the ovary; (2) the ova are fertilized, forming brood *C*, and the older brood, *A*, is born at practically the same time; (3) broods *B* and *C* develop for a period of 10 to 22 days and during this time another group of younger ova develop to the maximal size; (4) brood *B* is born, brood *C* is retained in the ovary and the eggs of the new group are fertilized to form brood *D*.

Superfetation, as observed in the species mentioned above, is apparently caused by the earlier development of the younger ova, allowing earlier fertilization. A sequence might be arranged from the species described so far, illustrating regular stages through which superfetation might arise. (1) In *Gambusia* and *Mollienisia* the ova remain quite small until the birth of the brood already contained in the ovary; they may not reach a state in which they can be fertilized for about two weeks. (2) In *Lebistes* the developing ova are much larger and better developed when the brood in the ovary is born and this continued growth brings them to the point of fertilization a few days earlier. (3) In *Quintana atrizona* the ova have reached a still more advanced stage at the birth of the brood in the ovary; they have reached their maximal size and complete differentiation and fertilization takes place immediately. (4) The ova in *Pœciliopsis infans* and *Pœcilistes pleurospilus* attain their full size and complete differentiation long before the brood in the ovary is born. Fertilization of the ova takes place and the resulting embryos are retained in the ovary as a second brood.

Heterandria formosa.—This species has small broods with short intervals between the broods.

The records shown in Table III are those of two young females that have just attained sexual maturity. The most important features in the records are: (1) the broods are very small, one or two embryos being produced at a time during the winter months and as many as five or six at one time during May and June; (2) the interval between broods is long during the winter months, thirty-three to forty-one days. It becomes shorter with the approach of spring, reaching the shortest interval —three to eight days—in May and June. Seal (1911) made a record of broods produced by larger specimens during the months of July and August. He found that the broods containing from four to sixteen embryos were born at intervals of from four to nine days.

A female which had produced a brood of nine was sacrificed and examined on May thirteenth. The ovary was well filled with embryos and when these were dissected out of their follicles and arranged in groups it was found that six levels of development could be distinguished. There were nine embryos at the oldest stage (brood *A*) and these would presumably have been born about seven days later. All external parts of the embryos were well developed except the caudal fin, and the yolk sac was almost completely absorbed. They were about 5 mm. in length (snout to base of caudal fin). Eight embryos formed

Table III

Brood production in *Heterandria formosa*

Specimen No. 1

Date	No. of young in brood	Interval in days between broods
December 12	1	
January 24	2	33
February 9	2	16
March 1	2	19
March 17	2	16
March 29	3	12
April 6	2	8
April 14	3	8
April 26	2	12
April 30	3	5
May 4	2	4
May 12	5	8
May 20	3	8
May 26	2	6
June 2	3	7
June 8	2	6
June 14	3	6
June 17	5	3
June 20	4	3
June 28	3	8
July 1	1	5
July 11	2	10
July 25	1	14

Specimen No. 2

Date	No. of young in brood	Interval in days between broods
December 10	2	
January 21	2	41
February 7	2	17
February 28	2	21
March 6	1	6
March 17	3	11
April 4	2	18
April 7	2	3
April 20	2	13
April 26	3	6
April 30	1	4
May 9	3	9
May 12	2	3
May 20	2	8
May 26	6	6
May 31	4	5

brood *B*. The members of this brood were considerably younger than those forming brood *A*. There were eight embryos in brood *C*, nine in brood *D*, eight in brood *E* and at least six in brood *F*. It is possible that a still younger brood was present. The largest unfertilized cells were about .7 mm. in diameter and it is evident that fertilization would occur when the cells had reached this size.

The only peculiarity in the history of the ova up to the time of fertilization lies in the fact that they are very much smaller at fertilization than the ova of the other species described. This fact would indicate that the larger quantity of yolk elaborated in the ova of the others is not required in *Heterandria formosa*. If the larger amount of yolk is not formed and yet the embryos go on to the more advanced stage while being retained in the ovary, it must be assumed that the follicle cells surrounding the developing embryos are responsible for furnishing the food materials. The expanded yolk sac, containing little yolk, is in a position to absorb the food materials, for it completely surrounds the embryos in the earlier stages, becomes very vascular, and is in contact with the walls of the follicle almost up to the time of birth.

Both viviparity and superfetation in this species have been carried to an extreme degree and it is quite likely that there will be found in some small pœciliïd fish a stage intermediate between that of *Heterandria formosa* and that represented by *Pœciliopsis infans* and *Pœcilistes pleurospilus.*

It is reported by Stoye (1935) that the mode of reproduction in *Priapella bonita* and in *Phalloptychus januarius* is similar to that in *Heterandria formosa*, one or two young being born at a time at intervals of a few days.

It is also reported by Stoye that in *Micropœcilia branneri* the young are born one or two at a time every few days. This observation would tentatively class this species with *Heterandria formosa* in its mode of reproduction. A most interesting situation is presented in this case since it has been stated by Stoye and verified by the writer that two other species in this genus (*M. paræ* and *M. picta*) produce broods of thirty to forty embryos at intervals essentially like those of the *Lebistes* type. Unfortunately no breeding specimens of *Micropœcilia branneri* have been available and gravid ovaries have not been examined.

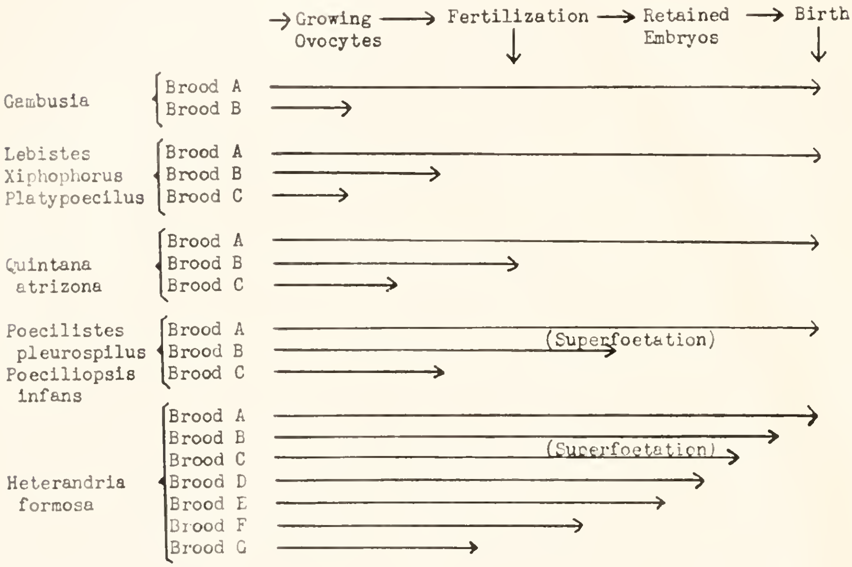
SUMMARY

A graphic summary of the relations of growing ovocytes, mature ova, the fertilization process, the broods of embryos retained in the ovary and the time of birth is offered in Table IV. Each group represents the situation within a single ovary at the height of the reproductive season and at the moment in which a brood is born. The term brood is used to indicate a group of growing and differentiating ovocytes of approximately equal development up to the time of fertilization and also the embryos produced by the fertilization of these ova up to the time for birth. No quantitative relations are implied concerning comparative size of ovocytes or embryos in different species nor of comparative lengths of time that embryos are retained in the ovary.

A study of Table IV leaves one with the impression that the extreme type of superfetation in *Heterandria formosa* might have arisen from the simpler type as illustrated in *Pœcilistes pleurospilus*. The argument also appears to be defensible that the simpler type of superfetation found in *Pœcilistes* might have arisen from a condition like that illustrated by *Quintana atrizona* in which a new group of ova are ready for fertilization when a brood is born but in which fertilization is delayed for some hours. All of this implies that the extreme cases of superfetation should be considered as the specialized and derived types while those without superfetation are the simpler. Since the Pœciliidæ undoubtedly arose from an oviparous cyprinodont such an assumption does not seem to be overdrawn. That group of the Pœciliidæ (*Gambusia* and *Mollienisia*) with the ovarian cell cycle which most resembles the oviparous cycle may by the same sign be considered the most unspecialized.

The evidence which now exists concerning the evolutionary relations of the different genera of the Pœciliidæ gives no support to the thought

Table IV


Growing Ovocytes
Fertilization
Retained Embryos
Birth
Gambusia
Brood A
Brood B
Lebistes
Xiphophorus
Platypoecilus
Brood A
Brood B
Brood C
Quintana atrizona
Brood A
Brood B
Brood C
Poecilistes pleurospilus
Poeciliopsis infans
Brood A
Brood B
Brood C
(Superfoetation)
Heterandria formosa
Brood A
Brood B
Brood C
(Superfoetation)
Brood D
Brood E
Brood F
Brood G

that those genera in which superfetation occurs are more closely related to each other than to any other genera. Rather, it appears that superfetation has arisen more than once and that the present cases represent convergent evolution. The occurrence of superfetation therefore has no taxonomic significance.

The Control of Reproductive Cycles

The factors which control the rhythms of reproduction in this group of fishes are presumed to be the same as for other fishes and are probably also the same group of factors which control the cyclical production of ova in higher vertebrates. These factors would be in part environmental, in part the driving influence of the gonad-stimulating hormones of the pituitary and, finally, an hereditary factor which would place a limit within which the others might act.

Annual Rhythm

In most of the laboratory-reared pœciliïds in which an annual record has been kept, it has been noted that the number of embryos in a brood and also the number of broods produced increases in early spring, reaches a climax in late spring and early summer and declines in late summer. Production of young falls to a low level or ceases altogether during the winter. Studies on *Gambusia affinis* by Barney and Anson (1921*a*), Hildebrand (1917) and Kuntz (1913) show that the same type of rhythm is present in specimens studied in the field under natural conditions. In the laboratory specimens reared by the writer, food and temperature have been maintained at a constant level. In the natural habitat, both temperature and food supply have some effect on the rhythm. The laboratory-reared specimens have been subjected to one variable factor, namely light. No attempt has been made to regulate the length of time each day during which specimens were exposed to light and consequently they have been exposed to the usual seasonal fluctuation. The highest point in the reproductive cycle coincides with the longest daily exposure to light and the lowest point in the reproductive rhythm to the shortest daily exposure. This coincidence would seem to be significant in view of the fact that seasonal variation in light has accompanied seasonal variation in the reproductive cycle in both wild and laboratory specimens. It might be tentatively postulated that light acts directly to stimulate the reproductive organs differentially but more likely through the gonadotropic hormones of the anterior pituitary.

Brood Production

Apparently the factors which control the size of broods and the rate of brood production are in part the same as those concerned with the high and low phases of the annual cycle. The contrast between the reproductive activity exhibited by an ovary of *Heterandria formosa* at the height of the reproductive season and one at a low ebb is striking and demonstrates clearly one of the factors responsible for brood production. In the ovary at the height of the breeding season there are five or six broods of several individuals each at different levels of development. At a still lower level are one or two groups of cells growing to the size necessary for fertilization. Every few days a brood is born and all levels of embryos and younger fertilized cells move up to a higher level. The entire picture is one of the crowding up of the maximal number of cells from the lower levels with a stimulating factor driving them, with the result that larger broods are produced and the intervals between them decreased. The reverse of this picture is seen in the ovary of specimens of this same species during the winter. Small numbers of ovocytes are developing and if broods are present in the ovary there are rarely more than two and they consist of one or two embryos each with a wide gap between them in level of development. The impression is created that the cause for the infrequency of broods, the small numbers of embryos in broods, and the paucity of developing ovocytes, lies in the lack of a stimulating agent. As suggested before this agent is probably a follicle-stimulating hormone from the anterior pituitary.

An influence retarding the development of the younger ovocytes is also present. This is best seen in the ovarian activity in species without superfetation. In *Gambusia*, for example, the younger ovocytes will develop to a diameter of only .3 mm. until the brood of embryos held in the ovary is released. Then within a few days they more than triple their diameter. In *Lebistes* and *Xiphophorus* the retarding effect is not so marked and ovocytes become much larger (0.8 mm. to 1.0 mm.) before a brood is discharged. In *Quintana atrizona* retardation by the growth of embryos is still less and the ova reach the maximal size and are ready for fertilization before the previous brood is evacuated. However, the retention of a brood of embryos seems to act as a block upon fertilization; otherwise some cases might be found in which the fully matured ova had been fertilized before the discharge of the older brood of embryos. Although the block upon fertilization of a new group of ova exercised by the presence of a brood of embryos seems to be removed in *Poecilistes pleurospilus*, it is apparently partly operative for not more than two levels of broods are found in the ovary at one time.

The presence of older ova also serves to retard the development of younger cells. In an old virgin female a group of ova will have reached the maximal size and younger groups of cells approximately equal in numbers will be found at lower levels. If fertilization is prevented, the younger groups of cells will not develop and the condition may prevail for months. When the older group of ova is fertilized and the embryos begin to develop, the block is released and when the embryos are discharged the younger waves of cells will advance.

From the above observations the controlling mechanism might be postulated as follows: (1) a hormone stimulates the ovary to produce waves of developing ovocytes. The production of the hormone or its effectiveness is acted upon by light and possibly other factors in the environment. (2) A retarding influence upon the growth of younger cells is exercised by the presence of older cells and by broods of retained embryos. (3) A balance has been struck between the two influences and is fairly constant within a species so that the rate of brood production at any season is practically constant. Hereditary factors control this balance. (4) The balance between the stimulating and retarding influences varies with the season, the stimulating agent being most effective in spring and early summer and least operative during the fall and winter months.

Relation of Broods to Annual Cycles in Other Fresh Water Fishes

Some of the fresh water fishes of the temperate zones have annual life cycles, maturing their gametes within the year and dying before another season of reproduction. This type of life cycle, described by Hubbs (1921) in *Labidesthes sicculus,* is not common. Other fishes bring their ova to maturity at the end of their second or third year or later and a new generation of ova to maturity each year thereafter as long as they live. In some fishes the ova have been two years in coming from ovogonia to the full size and complete differentiation necessary for fertilization. *Cottus bairdii,* described by Hann (1927), represents this type. It has been observed by the writer that *Perca flavescens* in Lake Michigan follows the same course in developing its ova although there is evidence that *Perca* requires sometimes three years before the first batch of ova is matured. In these fishes of the temperate zone a marked retarding influence is exerted by the cold weather of late fall and winter. Under more favorable food and temperature conditions such as can be maintained in the laboratory, an earlier maturing of gametes can be secured indicating that the reproductive rhythm occurring in nature is not the maximal one. These same fishes under tropical conditions would presumably produce more young in a shorter space of time although inherent factors would limit the rapidity of the occurrence of cycles.

As already indicated, the Pœciliïdæ arose in a tropical climate (Eigenmann, 1906) and some genera have migrated into the north and south temperate zones. Their pattern in reproductive cycles has apparently been fixed in the tropics under most favorable conditions for the production of the maximal number of cycles in the shortest space of time. Apparently other inherent factors associated with viviparity have had some influence in shortening reproductive cycles as oviparous fishes like the cichlids, which have been associated for a long time with the Pœciliïdæ in geographic distribution and climatic conditions, do not have such short cycles.

The production of successive waves of ovocytes in the Pœciliïdæ is interpreted as parallel to the seasonal waves produced in such forms as *Cottus* and *Perca*. It takes two years to produce a batch of mature ovocytes in *Perca* and *Cottus* and only a few months in the pœciliïds. If any pœciliïd should produce a single batch of ova, bring them to maturity and fertilization and void the embryos without bringing any more ovocytes to maturity, we should have a case parallel to that of *Labidesthes,* but apparently none exists. The interpretation of each pœciliïd short cycle as equivalent to the long cycle of *Cottus* and *Perca* instead of a long-drawn-out annual cycle, as suggested by Barney and Anson and Kuntz, rests upon the question of whether there is synapsis in residual gonial cells on a large scale to produce the entire season's potential ova or synapsis of only enough residual gonia to form the ova for a single brood. If synapsis occurred on the necessary large scale and only once a year, followed by the maturing of small groups of ovocytes, an annual cycle such as occurs in *Cottus* and *Perca* might be postulated. However, during the height of the breeding season small waves of synapsis of gonia occur at regular intervals and new ovocytes are being added at the lower levels as the ones at the upper levels reach maturity and are fertilized.

SUMMARY

1. All species of the Pœciliïdæ are ovo-viviparous.

2. At the height of the breeding season broods are produced at regular intervals varying from about forty-five days in some species to five or six days in others.

3. In laboratory-reared specimens and in specimens breeding in temperate zones the shortest interval between broods occurs in spring and early summer and the longest interval during fall and winter.

4. In the reproductive cycle of *Mollienisia* and some other species, the oldest group of ovocytes remaining in the ovary just after a brood has been extruded is very small. Growth is rapid and the ova are ready for fertilization about eight days after the voiding of the last brood of embryos.

5. In *Lebistes* and other species with similar reproductive cycles, the oldest group of ovocytes remaining in the ovary just after a brood has been voided are much larger than in *Gambusia* and fertilization occurs a few days earlier than in *Gambusia*.

6. In *Quintana atrizona* the oldest group of ova remaining in the ovary are fully developed and ready for fertilization at the time of birth of the previous brood of embryos.

7. Superfetation occurs in *Pœcilistes pleurospilus* and several other species. Fertilization of the oldest group of ova takes place before the voiding of the brood already in the ovary and two broods at different levels of development are found in the ovary.

8. In *Heterandria formosa* and possibly other species there is an extreme development of superfetation with six or more small broods at different levels of development occurring in the ovary at one time at the height of the breeding season.

9. Older groups of ova and embryos retained in the ovary retard the development of younger groups of cells and a balance is maintained between this retarding influence and the agent, assumed to be a follicle-stimulating hormone from the pituitary, which forces the cells to grow from the lower to the upper levels of development.

10. The short reproductive cycles of the Pœciliidæ are the equivalent of the longer annual or biennial reproductive cycles of the fishes of the temperate zones.

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THE EXPERIMENTAL DECOMPOSITION AND REGENERATION OF NITROGENOUS ORGANIC MATTER IN SEA WATER[1]

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HISTORICAL

Although the formation of nitrate from composted nitrogenous organic materials has been long known and practically applied, the current conception that specific biological agencies bring about these natural processes in soils, fresh waters, and the sea was developed only in the last quarter of the nineteenth century. In his studies on acetic acid fermentation Pasteur (1862) anticipated the rôle of bacteria in the ultimate oxidation of ammonia to nitrate, but Schloesing and Müntz (1877 et seq.) first demonstrated the biological nature of the intermediaries. The demonstration was performed in soils; Müntz, shortly afterward (1890), showed that ammonia was produced in soils by microbial breakdown of proteinaceous organic matter. That the oxidation of ammonia to nitrate proceeded through nitrite was distinguished by Munro (1886), but not until Winogradsky (1891, 1892) performed his classic researches were the two oxidations recognized as separately determined by specific ammonia-oxidizing and nitrite-oxidizing bacteria.

Following these researches Adeney (1895) demonstrated the complete sequence of nitrate regeneration in natural and sewage polluted water, establishing the steps by clear-cut quantitative methods. His procedures determined the basis for standard sewage analysis.

It has been held by Brandt (1899, 1902) and many others that the cycle of nitrogen in the sea follows essentially the same phases, and, using this interpretation, a number of researches into the occurrence and distribution of specific marine organisms have been made. These investigations have been reviewed and extended by Waksman, Hotchkiss and Carey (1933). There is considerable conflict in the literature on biological nitrification in the sea, but the conventional picture derived from studies of soils and fresh waters is nevertheless applied to oceanographic chemical data.

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[1] Contribution No. 129 of the Woods Hole Oceanographic Institution.

EXPERIMENTAL

Because of the growing interest in the regeneration of nitrate in the sea it seemed highly desirable to make a quantitative investigation of the course of organic decomposition and of the simultaneous appearance of nitrogenous products in the water. To this end, a number of experiments were carried out in which a natural source of organic matter—plankton material from customary net-hauls—was allowed to rot and

TABLE I

Series I. Source of organic matter: mixed plankton tow.
Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
6–27....	188	0	0	5	5	5	193	150	100
29....	150								160
30....		80	0	5	5	85			204
7– 2....	131	107	0.1	5	5	112	243		180
5....	110	160	3.8	6	2	166	276		123
10....	82						277		
11....		185		10		195			22
15....			2.4						
17....	77						308		40
18....		225	2.7	6	3	231			
24....	40		16				272		
25....		200		32		232			20
27....			55	70	15				
28....		185	80	90	10	275			
30....		125	110	145	35	270			36
8– 1....		60	180	215	35	275			
5....		11	150	275	125	286			
7....	47	0	110	300	190	300	347		
10....		8	2.2	300	298	308			
8–11*....		Portion inoculated with diatoms							
19....	285	0	0	20	20	20	305		
22....	300	0		13	13	13	313		
8–11*....		Control portion, not inoculated							
12....			0.5	360	360				
14....				330					
17....			0.6	330	330				
19....	37	0		360	360	360	397	200	
21....			0.8	330	330				
22....		0							

* One portion inoculated with diatoms and placed in the light.

‡ Does not include dissolved organic nitrogen.

decompose in sea water. Observations were made to follow the disappearance of nitrogen in the decomposing material and its appearance in the water in the form of ammonia, nitrite and nitrate, in the effort to reproduce artificially the cycle of organic decomposition and eventual regeneration. If, by means of the soluble compounds resulting from the decomposition, the water could be rendered fertile and capable of

TABLE II

Series II. Source of organic matter: mixed plankton tow.
Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system ‡	Dissolved organic N	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water			
7-12....	400	18	0.2	8	8	26	426	420	82
14....	345								176
15....		>120	0.5	8	8				265
17....	275	>120	0.2	6	6				270
20....	202	550	2.3	8	6	558	760		80
23....		580							
24....			16						43
25....		600		27		627			
27....			60	80	20				
28....		650	110	150	40	800			25
29....	90								
30....		550	240	300	60	850	940		
8- 1....		340	550	590	40	930			16
5....		12	800	820					
7....	78	4	850		60	914	992		
8....				910					
10....		8	550	1000	450	1000			20
12....			190	1100	910	1100			
14....		35	0.1	1050	1050	1100			25
17....			0.1	1100	1100				
18....	133						(1250)		
8-21*....		Portion inoculated with diatoms							
27....	305								
28....			1.0						
31....	840		7.7	320	312	(330)	(1170)		
9- 2....	1010		0	25	25	(35)	(1045)		
3....								470	
8-21*....		Control portion, not inoculated							
22....		7	0.5						
28....			0.3	1050	1050				
29....				1000					
31....			0.5						

* One portion inoculated with diatoms and placed in the light.
‡ Does not include dissolved organic nitrogen.

supporting a new organic growth, comparable to that previously decomposed, the complete cycle would have been carried out. It was also hoped that periodic chemical analysis, by methods now available, might throw light on the sequence of the various steps in the cycle.

The raw material was mixed plankton, collected in the usual way in a No. 20 net, quickly washed, and kept on ice for the few hours before it could be returned to the laboratory. This material was then suspended in 10–15 liters of fresh, filtered sea water and stored in the dark at a temperature of 20–25° C.

Several different analyses were carried out on this material at the start and at intervals thereafter, the various fractions being determined as follows:

1. Total nitrogen contained in the suspended or particulate matter. This is called "plankton nitrogen" in the following descriptions, but also includes bacterial nitrogen as well as that in any other form of suspended matter. This determination follows the procedure described by von Brand (1935) and consists essentially of precipitating the

TABLE III

Series IV. Source of organic matter: mixed plankton tow, strained through No. 8 bolting silk. Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system *	Bacteria in thousands per ml.
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *		
7-28	115	43				55	170	152
29			0.6	12	12			200
30	83	110						305
8- 1	63	150	1.0	15	14	165	228	190
5	38	205	<1	10	10	215	253	35
7		190	0.6	10	10	200		16
10	40	200		15	15	215	255	
14		240	0.5	20	20			25
17	41			17	17			
20			13					
21				35				20
22		210	21			(250)		18
27	58	175	120	140	20	315	373	
29			190					
31		29	220	240	20	269		
9- 2		25	225	225	(0)	250		
5	59							
8				260				
14		14	310	330	20	344	(400)	
27			130	290	160			
10-4			1					

* Does not include dissolved organic nitrogen.

suspended matter in a sample of the water by the addition of alkali. The precipitate drags down all suspended and colloidal matter, and after settling and centrifugation is separated and resuspended. The resulting small volume of suspension is then used for a determination of total nitrogen by the method of Krogh and Keys (1934): fusion with KOH in a stream of hydrogen, followed by recovery of the ammonia.

2. Ammonia in the water, by a slight modification of the method of Krogh (1934), in which the original design of the still was changed somewhat, to make it more compact and to permit heating electrically.

3. Nitrite in the water by the well-known Griess-Ilosvay method.

4. Nitrate (including nitrite) in the water, by Harvey's reduced strychnine method. Nitrate then calculated by subtracting the value of nitrite as obtained under 3.

TABLE IV

Series VI. Source of organic matter: washed, persisting culture of *Nitzchia*. Micrograms of nitrogen per liter.

Date	Plankton	In water					Total in system *
		Ammonia	Nitrite	Nitrate + nitrite	Nitrate	Total in water *	
8–14			0.1				
15	633	2		17	17	19	652
18	514	120	0.1	15	15	165	679
21	430	230	0.1	16	16	246	676
27	298	310	0.4	24	24	334	632
9– 2	201	410	1.3	20	19	430	631
8	226			25	25		
14		490	0.1	17	17	507	(700)
27			0.2	20	20		

* Does not include dissolved organic nitrogen.

5. Bacterial counts.

The data from the chemical analyses are collected in Tables I to IV and some of them are also presented graphically in Figs. 1 and 2.

Several different "series" were carried out: I, II and IV, in which the organic material was a mixed tow of both phyto- and zoöplankton, of varying amount in each case; and VI, in which a persisting washed culture of diatoms was used as the source of organic matter. In Series II the sea water in which the plankton was suspended came from the surface of Woods Hole Harbor, which probably accounts for its higher content of dissolved organic nitrogen than that of the water in the other series, which was obtained from the surface a few miles to the south of Marthas Vineyard.

CHEMICAL

The rate of decomposition of the plankton varied in the different series, but after an interval of from 8 to 20 days decomposition stopped. At this time there was still a constant and not inconsiderable amount of nitrogen still remaining, either in non-decomposable plankton residues, bacterial cells, or in other forms. The amount of this residual nitrogen was from 20 to 35 per cent of that originally determined in the plankton, or 7 to 10 per cent of that calculated by addition of the different soluble and insoluble nitrogen fractions.

The rate of decomposition is greatest in the first few days. In Series II, IV and VI it dropped rather suddenly, but in Series I it fell gradually over a longer period of time.

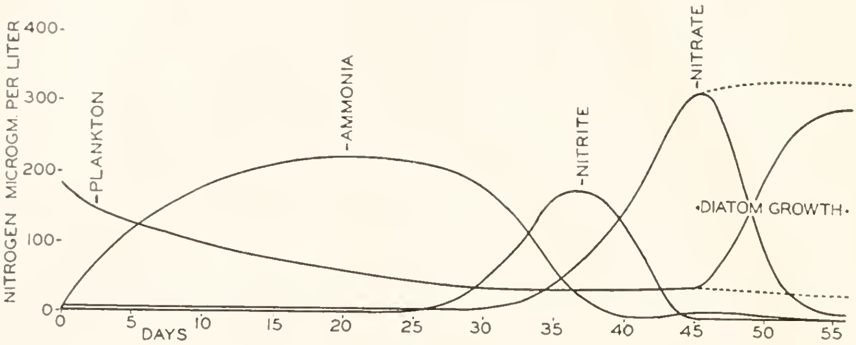

FIG. 1. Series I. The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended. One portion inoculated with diatoms on the forty-fifth day.

Ammonia appeared in the water rapidly and immediately at the beginning of the decomposition. Evidently the liberation of ammonia is involved in the very first steps in the degeneration of organic matter. It appears more slowly as time goes on, and reaches a maximum when the plankton decomposition ends. During this time there is no rise in the nitrite or nitrate in the water.

The disappearance of ammonia is accompanied by the appearance of nitrite in the water, and this in turn is eventually oxidized to nitrate. The phase during which nitrite was present was 15–20 days in Series I and II, but extended to 40 days in Series IV. In any event, it is not until nitrite reaches its maximum and starts to disappear that nitrate increases.

Eventually, however (after 45 days in Series I, 40 days in II, and 65 days in IV), all the available nitrogen was oxidized to nitrate. At this point, in both Series I and II, a portion of the water was placed in the light and inoculated with fresh diatoms, which grew rapidly, raising the "plankton nitrogen" again and lowering the nitrate to its original minimal value. In this way the complete cycle was carried out.

The question of the existence of soluble nitrogen compounds intermediate between plankton material and ammonia could not be definitely answered, but there was no evidence of any such. Anything in the nature of colloidal cell-material, partially decomposed, must have been carried down in the precipitation and determined with the rest of the plankton nitrogen.

In the transformation of ammonia to nitrite and nitrate it seems that we are dealing not with an equilibrium of simultaneous processes but with distinct, consecutive steps, each doubtless determined by its own set of conditions.

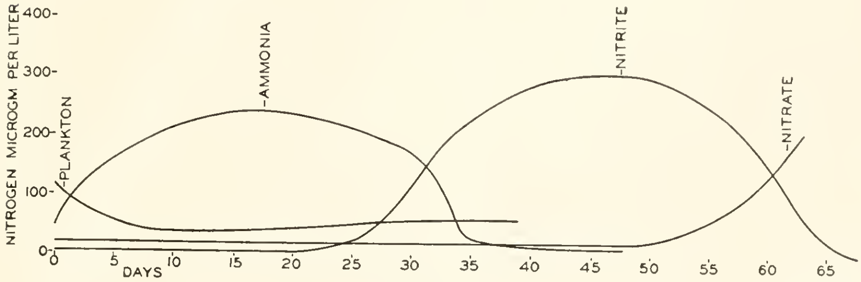

FIG. 2. Series IV. The decomposition of nitrogenous organic matter in mixed plankton, showing the appearance of soluble nitrogen compounds in the water in which it is suspended. Plankton previously filtered through No. 8 bolting silk.

A peculiar situation is revealed by the data from Series I, II and IV. The analyses indicate a larger amount of nitrogen regenerated in soluble forms in the water than that lost by the plankton during decomposition. In the tables the "total nitrogen in the system" has been calculated by addition of all the separate forms determined. This total shows in general a continuous increase throughout the decomposition, especially in Series II. That it is not observed in Series VI may be in some way connected with the fact that the organic material in this case consisted of diatoms.

There are three possible explanations for this nitrogen discrepancy:

1. Participation of other forms of nitrogen in the cycle—most plausibly the dissolved organic nitrogen in the water.

2. Systematic errors in either the sampling procedure or the analytical method, so that the values for plankton nitrogen are regularly too low.

3. Nitrogen fixation.

Each of these possibilities has been critically examined and at the present time none of them seems an altogether acceptable explanation. Determinations of the dissolved organic nitrogen in the water, by the method of Krogh and Keys, did not show any significant change during the experiments. Filtering out the larger plankton organisms in Series IV, to lessen the chance of irregular sampling, had no effect. On the other hand, it is true that the decomposition of very much more finely divided organic matter (diatoms) in Series VI yielded only an equivalent amount of ammonia, but this experiment had to be stopped before nitrification began, which is usually the time when the most pronounced difference between added and recovered nitrogen appears.

According to currently accepted views of biological fixation of nitrogen the third possibility seems doubtful.

A further study of this nitrogen discrepancy is now under way.

BACTERIOLOGICAL

The regeneration of inorganic nitrogen from its various organic forms in plankton proceeds through four recognizable stages, each of which is incident to the development and activity of a special bacterial flora. Following the death of plankton organisms, brought about in these experiments by handling, unfavorable temperatures, lack of illumination, crowding, and inadequate nutrient supply, the more labile components of the dead cells undergo hydrolytic cleavage, simultaneously liberating a fraction of the amino nitrogen as ammonia. This breakdown, it would appear from the rate at which ammonia appears and plankton nitrogen diminishes, is not analogous to the digestion of protein by higher animals where individual protein complexes are first split into smaller molecules bearing amino nitrogen.

A part of the decomposition may be autolytic, but it is evident from the tables, which show an attending rapid development of various species of bacteria, that these organisms must play a large part. The absolute numbers are never very high—in fact, not greater than would be expected in freshly taken, unfiltered sea water stored for such intervals of time (Waksman and Renn, 1936; ZoBell and Anderson, 1936). The decline in plankton nitrogen is not so precipitous as the decrease in bacterial numbers. This may mean that the various fractions of plankton nitrogen are not equally susceptible to bacterial attack.

Liberation of ammonia from plankton and other nitrogenous organic matter is, bacteriologically speaking, unspecialized, but is dependent upon the relative proportions of available nitrogen and carbon in the decomposing materials—the materials used in these experiments are, as Redfield (1934) has shown, characteristically nitrogen-rich.

Following the first burst of bacterial activity the ammonia concentration reaches a high level capable of supporting an active nitrite-forming population. Unsuccessful attempts were made to follow the development of the specific bacterial flora responsible for the oxidation of ammonia to nitrite. It appears from the many investigations upon nitrite-forming bacteria in the sea, that the process is due to specific organisms of the *Nitrosomonas* group analogous to the specialized nitrite-forming flora of soils (Waksman, Hotchkiss and Carey, 1933; Carey and Waksman, 1934). The rate at which nitrites are developed here is roughly the same as that observed in cultural studies of the process. Crude cultures prepared by inoculating sea water containing ammonium salts with mud or sea water usually give positive tests for nitrite within ten days or two weeks (depending upon the quantity of inoculum), after which the complete oxidation of ammonia to nitrite proceeds rapidly.

The final stage in nitrogen regeneration progresses more slowly, as is evident from Figs. 1 and 2. Nitrate formation is also effected by a specific bacterial flora, in this case, *Nitrobacter,* an efficient population of which seems to develop at a lower rate than either the non-specific ammonifying or specialized nitrite-forming bacteria. In culture experiments where crude inocula of nitrate-forming bacteria are added to sea water containing nitrite as the only sources of nitrogen, the period of incubation necessary to demonstrate measurable quantities of nitrate ranges from 30 to 90 days. Richer inocula bring about more rapid nitrate formation (Waksman, Hotchkiss and Carey, 1933).

It has been assumed that the relatively late appearance of nitrate in cultures made up of raw sea water to which organic nitrogen in some form has been added is due to the toxic effect of ammonia on nitrate-forming bacteria—a condition that has been clearly demonstrated in experiments on soils. But this relation, if it exists in these experiments, is masked by the slow growth of nitrate-forming bacteria even under favorable conditions. Thus in Series I and II the phase of nitrate formation began promptly after the disappearance of ammonia; in Series IV, on the other hand, it did not begin until 15 days after the ammonia had disappeared.

The regeneration experiments described here certainly are not identical with natural conditions and may not be expected to parallel exactly the courses of the individual phases of nitrate formation in the sea. On the other hand, they offer a clear picture of their relations. Further, they validate the conventional conception of the nitrogen cycle as derived from the various studies of the individual processes, by bringing them together in a single summary experiment.

SUMMARY

In conclusion, this study has shown:

1. That it is possible to reproduce the complete cycle of nitrogen regeneration.

2. That the transformation of decomposing plankton (especially diatoms) into ammonia is very rapid, beginning as soon as the initial substance disappears from the body. The amounts of soluble nitrogenous substances of higher molecular weight in these experiments can have been only very small.

3. The main stages in the decomposition are: dead body—ammonia—nitrite—nitrate.

4. Under these conditions, at least, no toxic substances are formed which inhibit the flowering of diatoms. The rapid and abundant development of the latter showed the regeneration of the water to be complete.

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STUDIES IN THE PIGMENTARY SYSTEM OF CRUSTACEA

II. Diurnal Movements of the Retinal Pigments of Bermudan Decapods

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Diurnal rhythms in behavior and physiological rhythms in certain organ systems of animals have long intrigued biologists and led to much interesting speculation as to the nature of the mechanisms involved in such activities. That the phenomenon is a general one is seen from the cases cited by Welsh (1930*a*). Such periodicity is most strikingly exhibited in the pigmentary changes of various animals, being reported in shrimps by Gamble and Keeble (1900), in brachyurans by Megušar (1912), in isopods by Menke (1911), in amphibians by Slome and Hogben (1929), in reptiles by Redfield (1918), and in cyclostomes by Young (1935). It is reasonable to expect that further study will reveal the existence of such rhythms in additional forms. Welsh (1930*a*, 1935, 1936) has shown that diurnal changes in the migration of the retinal pigments occur in several crustaceans even though the animals are maintained under constant conditions of illumination or of darkness.

In all the cases mentioned hormonal factors are involved to a greater or lesser degree in controlling pigmentary activity. Since the present studies (Kleinholz, 1937) are concerned with chromatic responses in crustaceans, only this branch of the general subject of color changes will be discussed here. The control of the bodily changes in color in crustaceans has been shown by Perkins (1928) and by Koller (1928) to be maintained by an endocrine which is liberated into the circulatory system from within the eye-stalks. Hanström (1935) and his associates (Sjögren, 1934; Carlson, 1936) have made histological studies of the eyes of many crustaceans, and have correlated the activity of the chromatophorotropic principle in eye-stalk extracts with the presence within the eye-stalk of a secretory structure which they call the *blood gland*. The mechanism that is involved in the control of retinal pigment migration in crustaceans is not yet completely known. Recent studies on *Palæmonetes vulgaris* (Kleinholz, 1936) present evidence for a humoral control of the distal and the reflecting pigments in the retina, but the proximal pigment appears not to be affected by the same eyestalk extracts which activate the first two sets of pigments. It is possible that one set of pigment cells is under nervous and the others under hormonal control.

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[1] The experimental work was carried on in Bermuda and was made possible by a grant from the James F. Porter Fund of Harvard University. I am indebted to Dr. J. F. G. Wheeler, Director of the Bermuda Biological Station, for placing the facilities of the Station at my disposal.

Before much progress can be made in analyzing the mechanisms by which diurnal rhythms are maintained under constant external conditions, it is advisable to study the phenomenon in a number of different species to determine which combinations of retinal pigments may be involved in such activities. This report on diurnal changes in the retinal pigments of several Bermudan crustaceans is offered to this end.

Materials and Methods

The various crustaceans used in these experiments were obtained by dredging at several stations in the vicinity of the Biological Station. After a sufficient number of animals of the same species had been brought into the laboratory, the specimens were divided into two groups, one of which was placed in a white porcelain bowl illuminated by a 40-watt electric lamp at a distance of 18 inches, while the second group was placed in a container in the dark-room. At least 12 hours were allowed for individuals to become adapted to light and to darkness.

After a period appropriate for adaptation, specimens were removed for fixation of the retinal pigments. For purposes of description the term "day-light" eye is used to indicate the retina of a specimen that was kept constantly illuminated and which was fixed or examined in the daytime, while "night-light" eye represents the condition of the pigments in an illuminated retina that was fixed at night; conversely, "day-dark" and "night-dark" are used to designate those specimens maintained in constant darkness whose retinal pigments were fixed during the day and at night, respectively. When a specimen was taken from the light-adapted group, a similar specimen was removed from the container in the dark-room for fixation at the same time.

Two methods of fixation for histological study were used. In one method, the animals were dropped into hot water (80° C.) for 10–20 seconds to fix the positions of the retinal pigments, and were then tranferred either to 5 per cent formalin or to a modified Bouin's solution containing 7 per cent acetic acid. In the second method the entire specimen, after the usual period of adaptation to darkness or to light, was dropped into a vial of Bouin's solution. The exoskeleton of most of the crustaceans contained large amounts of calcareous salts, but these troublesome deposits were dissolved by the acetic acid in the fixative, so that sectioning the eyes was greatly facilitated.

When the exoskeleton had been sufficiently softened by the fixative, the eye-stalks were excised and embedded in paraffin by a rapid dioxane treatment. After a preliminary rinsing of the excised eyes in water, the stalks were rolled over filter paper to remove any excess moisture and were then placed in full-strength dioxane over anhydrous calcium chloride. They were left in this fluid usually overnight (8–10 hours) and the next morning were placed directly into soft paraffin. The tissues were allowed to become infiltrated with the wax for about three hours, using two changes of paraffin, and were then embedded. No difficulty was encountered in cutting serial sections at 10 micra. Some

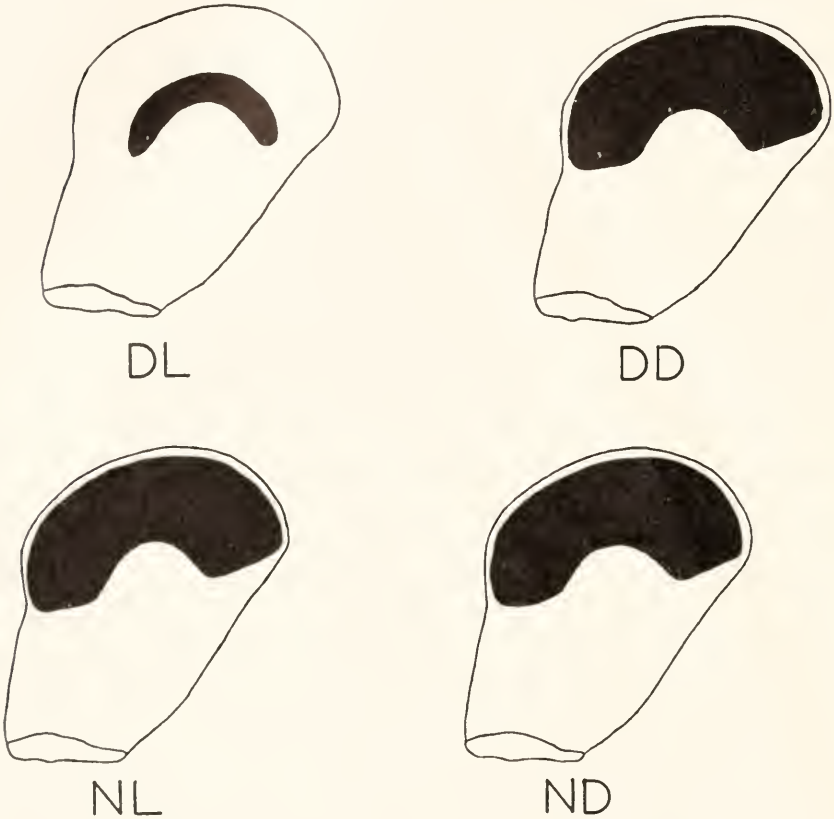


Fig. 1. Entire eye-stalks of *Eusicyonia,* viewed by transmitted light through the low powers of the microscope and showing the positions of the distal retinal pigment. *DL,* from a day-light animal; *DD,* from a day-dark specimen; *NL,* from a night-light individual; *ND,* from a night-dark shrimp.

sections were subsequently stained with Delafield's hematoxylin and eosin, and others were mounted unstained.

I am indebted to Mr. M. D. Burkenroad of the Bingham Oceanographic Foundation at Yale University, and to Dr. Fenner Chace, Jr., of the Museum of Comparative Zoölogy at Harvard University for identifications of the collected crustaceans. The two macrurans studied were *Eusicyonia n. sp.* (to be described by Mr. Burkenroad) and *Trachypeneopsis mobilispinis*, while the brachyurans were *Portunus anceps*, *Portunus depressifrons*, *Parthenope serrata*, and *Calappa flammea*.

Eusicyonia n. sp.

This new species of shrimp was taken from dredgings at 10–15 fathoms off Murray's Anchorage. Of the 12 specimens obtained for experimental study, 2 were males and the rest females.

While preliminary observations of the eyes of specimens kept under constant illumination showed that there was a change in position of the distal retinal pigment at night (Fig. 1), sectioning the eye was necessary to disclose the photomechanical changes of the remaining pigments. The eye of *Eusicyonia* is similar in structure to that of *Palæmonetes* as described by Parker (1897) and by Welsh (1930*b*). The positions of the three types of pigment cells are shown in Figs. 2–5.

An unusual situation was discovered in studying a number of sectioned eye-stalks of this species. Of the 12 specimens at hand, the retinas of 4 showed complete absence of reflecting pigment. The possibility that this might be a secondary sexual difference is eliminated by the fact that 2 of these shrimps were males and 2 females. The possibility that the difference is a fixation artifact can be ruled out by comparing eyes of specimens fixed by the two different methods. The reflecting pigment in the integument of many of the lower vertebrates is guanin, and is commonly found there in the crystalline condition (Ewald and Krukenberg, 1882). So far as I know, however, no studies have been made on the chemical nature of the pigments in the crustacean retina. If the retinal reflecting pigment is guanin, it might be slightly soluble in the acid components of the Bouin's solution. But the pigment was lacking only from those eyes which had been fixed with hot water and then preserved in formalin. This treatment does not seem to be sufficiently harsh to effect such changes. Moreover, of 4 specimens of *Trachypeneopsis* which had been fixed by the same method, the eyes of 1 lacked this reflecting pigment, while those of a second specimen were slightly deficient (Fig. 15); the retinas of the other 2 animals showed what may be called "typical" amounts. It seems more likely that the differences in amount of reflecting pigment are due to individual variations, possibly in the nucleoprotein metabolism of the animals, than to any fixation artifact.

On studying sections of the eyes of *Eusicyonia* fixed under the four experimental conditions (Figs. 2–5, 10–13) the following situation is found in the retinal pigments. The granules of reflecting pigment undergo no apparent changes in position in response to changes in light intensity. The main mass of the reflecting pigment is located on the distal face of the basement membrane; smaller amounts are found below the basement membrane and capping the distal ends of the distal retinal cells. The distal and the proximal pigments show readily discernible photomechanical changes:

Day-light eye

A. The distal pigment cells are to be found in the typical light-adapted state, the cells having moved proximally and come to rest against the proximal retinular cells.

B. The proximal pigment is also in the position typical for the light-adapted retina, much of it having migrated above the basement membrane to surround the rhabdome.

EXPLANATION FOR PLATE I

All figures are camera-lucida outlines to insure correct proportions, but the details have been drawn diagrammatically. *BM,* basement membrane; *DD,* day-dark retina; *DL,* day-light retina; *DP,* distal retinal pigment; *ND,* night-dark eye; *NL,* night-light retina; *PP,* proximal retinal pigment; *RP,* reflecting retinal pigment; *R,* rhabdome.

FIG. 2. Ommatidium from a day-light eye of *Eusicyonia.* The distal pigment lies against the proximal retinular cells, while much of the proximal pigment has migrated above the basement membrane to surround the rhabdome.

FIG. 3. Ommatidium from a day-dark retina of *Eusicyonia.* The pigments are in the typical dark-adapted position, the distal pigment having migrated distally towards the cornea, and the proximal pigment having moved completely below the basement membrane.

FIG. 4. Ommatidium from a night-light eye of *Eusicyonia.* The distal pigment is in the position characteristic for the dark-adapted retina while the proximal pigment is found above the basement membrane.

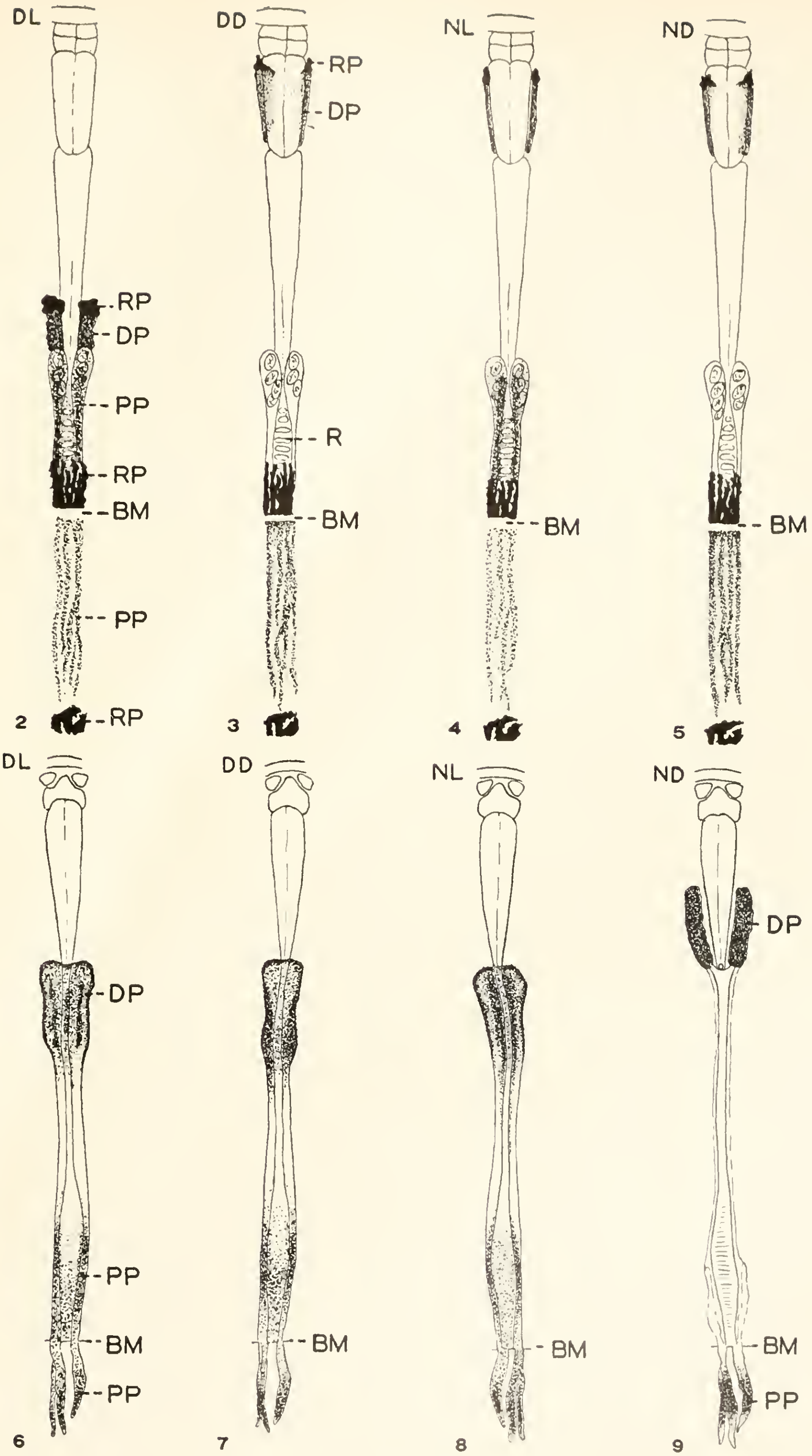
FIG. 5. Ommatidium from a night-dark eye. Both pigments are in the positions expected for a dark-adapted retina.

FIG. 6. Ommatidium from a day-light eye of *Portunus anceps.* The distal pigment is dispersed proximally toward the basement membrane, while much of the proximal pigment has migrated distally above the basement membrane.

FIG. 7. Ommatidium from a day-dark retina of *Portunus.* The positions of the pigments are the same as in the day-light eye, in spite of the fact that the animals were kept in constant darkness.

FIG. 8. Ommatidium from a night-light eye of *Portunus.* The positions of the retinal pigments are typical for what is expected in an illuminated eye.

FIG. 9. Ommatidium from a night-dark eye of the same species. The distal pigment has migrated distally, while the proximal pigment has moved proximally below the basement membrane.


DL
RP
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DL
DP
PP
BM
PP
6
DD
BM
7
NL
BM
8
ND
DP
BM
PP
9

Plate I

Day-dark eye

- *A*. The distal pigment is in the position characteristic for the dark-adapted retina, forming a collar around the distal ends of the cones.
- *B*. The proximal pigment has moved entirely below the basement membrane.

Night-light eye

- *A*. The distal pigment, in spite of the fact that the retina has been under constant illumination, is in the position typical for the dark-adapted eye, at the distal ends of the cones.
- *B*. The proximal pigment, however, is in the position found in the usual light-adapted eye, much of it having moved above the basement membrane.

Night-dark eye

- *A*. The distal pigment is in the position characteristic for a dark-adapted retina.
- *B*. The proximal pigment is also in the position found in the dark-adapted eye, having migrated completely below the basement membrane.

EXPLANATION OF PLATE II

Figs. 10–13 represent retinas of *Eusicyonia* in the four experimental conditions; Figs. 14–17 are photographs of similar retinas of *Trachypeneopsis mobilispinis;* Figs. 18–21 are photographs of the entire stalks of *Portunus anceps.* All the eyes are oriented so that the distal end of the retina is at the left and the proximal end to the right. *D*, distal pigment; *P*, proximal pigment; *R*, reflecting pigment.

FIG. 10. Day-light retina. The distal pigment has migrated proximally, the proximal pigment has moved above the basement membrane and lies directly to the right of the distal pigment, but the reflecting pigment is fixed.

FIG. 11. Day-dark retina. The distal pigment is seen to the left in its distal position, while the proximal pigment has migrated completely below the basement membrane.

FIG. 12. Night-light retina. The distal pigment is in the same position as in the day-dark retina, but the proximal pigment (which has been retouched in this photograph) is in the typical light-adapted position.

FIG. 13. Night-dark retina. The distal and the proximal pigments are in the positions characteristic for a dark-adapted eye.

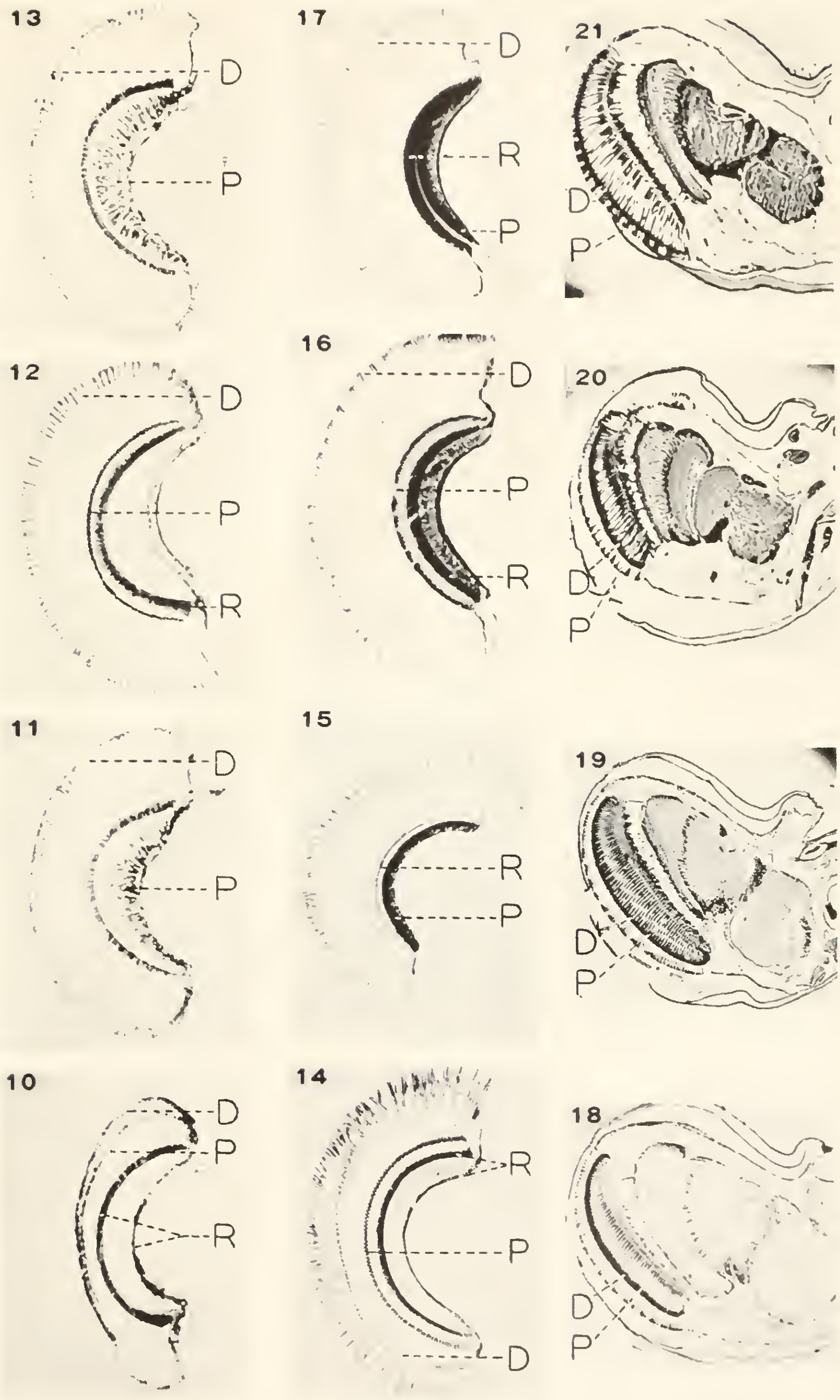
FIGS. 14–17 are comparable retinas of *Trachypeneopsis*, arranged in the same sequence as Figs. 10–13 and showing similar movements of the retinal pigments. Fig. 15 shows the very small amount of reflecting pigment in this retina compared with the others of this series.

FIG. 18. Day-light eye of *Portunus*. The distal pigment is the black band to the left. Granules of this pigment are dispersed proximally, while granules of the proximal pigment are dispersed distally towards the left.

FIG. 19. Day-dark eye. The positions of the two pigments are the same as in Fig. 18, in spite of the fact that the animals were maintained in constant darkness.

FIG. 20. Night-light eye. The pigments are in the positions characteristic for a light-adapted retina.

FIG. 21. Night-dark eye. The granules of the distal pigment have migrated distally from the processes in which they had been dispersed, while the proximal pigment granules have moved completely below the basement membrane. The blackness of the processes is due to the stain, and not to any contained pigment.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18
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Plate II

Trachypeneopsis mobilispinis

Crustaceans of this species were dredged off sandy bottoms in Castle Harbor and in Bailey's Bay. Eight animals were available for study; three of these were males and the rest females.

A particularly thick exoskeleton and dense pigmentation obscured the eye-stalk so that the positions of the distal retinal cells of whole eyes could not be determined by direct microscopic examination. Study of sectioned retinas, however, revealed a close similarity to the condition found in *Eusicyonia*, the chief difference being in the size relationships of the ommatidial components.

In this species, too, the reflecting pigment apparently undergoes no positional changes. In some specimens this pigment was completely absent, and in one only a trace of it was evident (Fig. 15). The retinal responses to light and to darkness are so similar to those in *Eusicyonia* that the same diagrams of Plate I (Figs. 2–5) serve to illustrate the changes. The distal and the proximal pigments show the usual positional changes in adaptation to light and to darkness. The resemblance to *Eusicyonia* in this respect is heightened by the fact that the persistent diurnal rhythm is found in the distal pigment cells, which, in night-light eyes, are in the position characteristic for a dark-adapted retina.

Brachyurans

Four species of brachyuran crustaceans were studied for the movements of the retinal pigments. Since the responses in all four were found to be identical, they may conveniently be described together.

EXPLANATION OF PLATE III

Figs. 22–25 are retinas of *Parthenope serrata* and Figs. 26–29 represent those of *Calappa flammea*. The retinas are oriented with the distal end at the top of the photograph and the proximal end toward the bottom. *D*, distal pigment; *P*, proximal pigment.

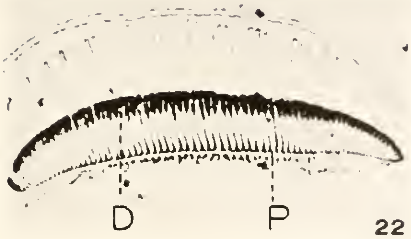
FIG. 22. Day-light retina. The distal pigment is dispersed proximally while the proximal pigment granules have migrated, for the most part, above the basement membrane.

FIG. 23. Day-dark retina. The positions of the two pigments are the same as in the day-light eye, in spite of constant darkness.

FIG. 24. Night-light retina. The pigments are in the positions typical for the light-adapted eye.

FIG. 25. Night-dark retina. The granules of the distal pigment have retreated distally, while those of the proximal pigment have withdrawn completely from the processes to a position below the basement membrane.

FIGS. 26–29 are photographs of retinas of *Calappa* arranged in the same sequence as Figs. 22–25 and showing a similar persistent rhythm in the day-dark eye.



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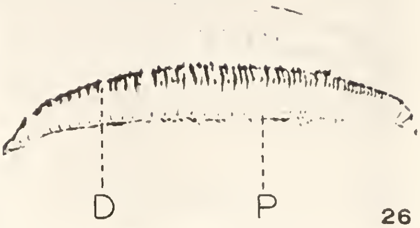

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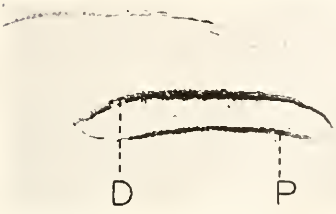

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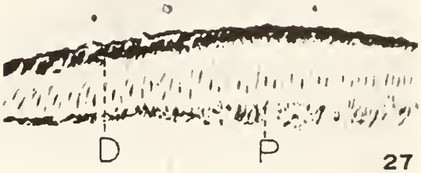

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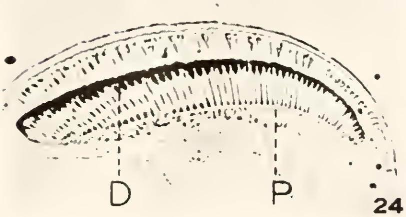

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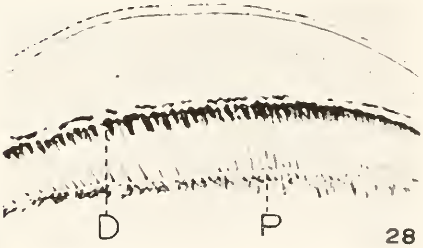

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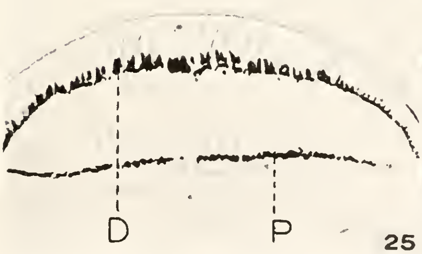

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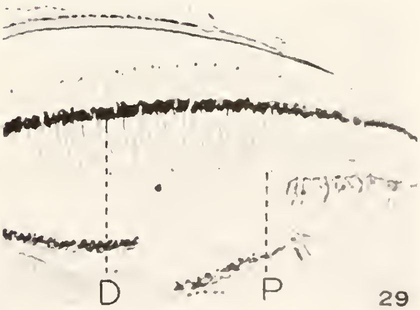

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Plate III

There are two types of black retinal pigments in these animals, a proximal and a distal set of cells; reflecting pigments seem to be completely absent. Diagrammatic illustrations of ommatidia are shown in Figs. 6–9.

The photomechanical changes of the retinal pigments in these brachyurans, although not as marked as those in the macrurans, are still evident:

Day-light eye
- *A.* The distal pigment is dispersed proximally toward the basement membrane.
- *B.* Most of the proximal pigment has migrated above the basement membrane and surrounds the rhabdome.

Day-dark eye
- *A.* The distal pigment remains in the position typical for a light-adapted retina.
- *B.* The proximal pigment also shows the persistent rhythm, remaining in the position found in a light-adapted eye in spite of conditions of constant darkness.

Night-light eye
- *A.* The distal pigment granules are in the same position as in the day-light eye.
- *B.* The proximal pigment is also in the light-adapted position.

Night-dark eye
- *A.* The distal pigment granules have retreated distally from the processes and lie in the cells which are located along the posterior region of the cones.
- *B.* The proximal pigment has moved completely below the basement membrane so that the processes of these cells are entirely free of pigment granules.

DISCUSSION

There is still much ignorance as to the nature of the mechanisms involved in these rhythmic activities. If the retinal pigments were uniform in their periodic responses under constant conditions, the problem as to the mediating agents would be relatively simple. But published accounts of the behavior of these pigments among the different crustaceans reveal perplexing heterogeneities in this respect. Examination of Table I will show the responses reported for various crustaceans.

In the first four species of macrurans, there is a uniformity of response in that the same set of retinal pigment, the distal, shows persistent periodic movements in the same direction under the same conditions of illumination. The next two species of shrimps are unusual in that they show evidence of possessing a double rhythm; not only are distal and reflecting retinal pigments involved in the periodic migration, but the periodicity occurs twice within a 24-hour cycle, once during the daytime when the animals are maintained in darkness, and once at night when the shrimps are kept under constant illumination.

Up to this point, the various responses, although still slightly confusing, are open to interpretation on a hormonal basis as found in the case of *Palæmonetes* (Kleinholz, 1936). The puzzling situations are those where the distal pigments show a persistent rhythm, but the reflecting pigments at the same time undergo only the normal changes in position characteristic for the particular condition of illumination. In *Leander tenuicornis* and in *Latreutes fucorum* the situation is reversed, the reflecting pigments showing the rhythm while the distal pigment cells respond only to differences in light intensity. If endocrine control of these two pigments is universally present in the crustaceans, such differences in response may possibly be due to threshold variations in reactivity to the same hormone, or, there may be two hormones involved, one for the distal pigment and one for the reflecting pigment.

TABLE I

Crustacean	Distal	Reflecting	Proximal	Investigator
Macrobrachium olfersii	*NL*	—	—	Welsh (1930*a*)
Macrobrachium acanthurus	*NL*	—	—	Welsh (1930*a*)
Eusicyonia n. sp.	*NL*	*Ab.* or *F*	—	This paper
Trachypeneopsis mobilispinis . . .	*NL*	*Ab.* or *F*	—	This paper
Leander affinis	*DD* and *NL*	*DD* and *NL*	—	Welsh (1935)
Anchistioides antiguensis	*DD* and *NL*	*DD* and *NL*	—	Welsh (1936)
Portunus anceps	*DD*	*Ab.*	*DD*	This paper
Portunus depressifrons	*DD*	*Ab.*	*DD*	This paper
Parthenope serrata	*DD*	*Ab.*	*DD*	This paper
Calappa flammea	*DD*	*Ab.*	*DD*	This paper
Leander tenuicornis	—	*NL*	—	Welsh (1935)
Latreutes fucorum	—	*NL*	*NL*	Welsh (1935)
Cambarus virilis	—	—	*DD*	Bennitt (1932*a*)
Peneopsis goodei	*F*	*F*	*DD*	Welsh (1935)

—, pigment shows normal photomechanical changes but shows no periodicity.

Ab., pigment is absent from the retina.

F, pigment is present, but undergoes no positional changes.

NL, rhythm in a night-light eye, the pigment moving into a typical dark position.

DD, rhythm in a day-dark eye, the pigment moving into the position characteristic for a light-adapted retina.

The mechanisms involved in the migration of the retinal pigments in the four brachyurans reported here is less open to analysis chiefly because so little is known about them. These crustaceans are the only ones thus far reported which show a pronounced periodicity in both the distal and in the proximal pigments at the same time. *Cambarus* and *Peneopsis* seem to fall into the same group in that the proximal pigment shows the rhythm.

The mediating agency for the migration of the proximal pigment is not known. In *Palæmonetes* the eye-stalk extracts which affect the distal and the reflecting pigments have no effect on the migration of the proximal pigment. Early workers on the migration of the proximal retinal pigment were of the view that this activity was under nervous control. But as Bennitt (1932*b*) states, the main argument against this belief is that no efferent nerve fibers have been found supplying these cells, their only nervous connection apparently being afferent fibers going to the optic ganglia (Parker, 1895). From the results of his experiments on the interrelation between the eyes of crustaceans (with regard to proximal pigment migration) Bennitt (1932*b*) believes that an endocrine control may be involved.

It is evident from this discussion that the possibility of formulating a general theory for the phenomenon of persisting diurnal rhythm is still remote. The fact that those crustaceans which have been studied lack the uniformity of behavior that is sought by the experimentalist, is probably sufficient proof of the complexity of the mechanism. In our present state of knowledge it can only be hoped that when the retinal pigments of other crustaceans have been studied with regard to hormonal control as in *Palæmonetes*, and the innervation of the proximal pigment cells studied by means of modern neurological techniques, a sufficient amount of information will have been gathered to attempt a general explanation of persistent periodicity in the retinal pigment migration of crustaceans.

SUMMARY

1. Persistence of a diurnal rhythm in the migration of the retinal pigments of several Bermudan crustaceans, in spite of constant conditions of illumination or of darkness, is reported. Two macrurans, a new species of *Eusicyonia* and *Trachypeneopsis mobilispinis*, show the distal retinal pigment in the position characteristic for the dark-adapted eye when the retinas of illuminated specimens are fixed at night.

2. Four species of brachyurans, *Portunus anceps*, *P. depressifrons*, *Parthenope serrata*, and *Calappa flammea*, show the distal and the proximal pigments in the typical light-adapted position when the specimens are kept in the dark-room and are fixed during the day.

3. The retinas of some of the macrurans showed deficiencies or absence of the reflecting pigment. This is thought to be due to differences in metabolism rather than to fixation artifacts.

4. Hormones are considered as the possible mediating agency involved in the phenomenon of persisting diurnal retinal rhythms.

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BACTERIA AND THE PHOSPHORUS CYCLE IN THE SEA[1]

CHARLES E. RENN

(From the Woods Hole Oceanographic Institution, Woods Hole, and the Biological Laboratories, Harvard University)

Marine bacteria, growing and multiplying rapidly under favorable conditions, are potential accumulators of the sea's labile organic matter. Lohmann (1911) has estimated that they may bind as cell substance each year from six to three hundred times the annual production of slower growing nannoplankton feeding upon them.

It is surprising, then, to discover, as Fischer (1894) and many others since have pointed out, that the bacterial population of the sea and large fresh-water lakes is very low—rarely exceeding a viable count of a hundred at the surface and extending through a lesser range in the depths (Birge and Juday, 1922; Reuszer, 1933). Lohmann found the mass of bacterial cells in sea water to represent an insignificant fraction of the nannoplankton; and it may be calculated, making generous allowance for non-viable cells, that the amount of phosphate, nitrate, or other nutrient substance bound by bacteria is immeasurably small. Thus, a million cells per cubic centimeter, at least a thousand times the likely bacterial population, would be equivalent to less than 3 mgm. PO_4 per cubic meter (Buchanan and Fulmer, 1933, pp. 69–71). Bacterial cell substance is notoriously rich in phosphorus, but it is apparent from their small numbers, that bacteria do not compete effectively for the sea's limited store of this element. Do these small numbers also indicate a low limit to their activity in the breakdown of debris and the regeneration of inorganic nutrients?

If Fischer's observations regarding the short life of marine bacteria are valid (he estimated their average viable span to be in the order of a half hour), then Lohmann's emphasis on their rôle as converters of organized material rather than accumulators gives a sound picture of their relations in marine economy. Rate of growth, length of the viable period, and rapidity of autolysis after death become critical factors in determining their value as regenerating agents.

Several experiments make it seem plausible that short-lived bacteria are active in phosphate regeneration, and offer confirmation for the smaller, inter-seasonal phosphorus cycles indicated in the investigations of Harvey and co-workers (1935) on plankton production, and remarked by Seiwell (1935). When specimens of sea water are stored in bottles at room temperature the bacterial population therein rises in two or three days to a level much higher, from a thousand to a hundred thousand times, than that of natural waters. This sudden increase is followed by a precipitous drop in viable numbers to a persistent low count (Waksman and Renn, 1936). That such unrestricted development of bacteria may withdraw appreciable quantities of phosphate from sea water is shown by the decreasing concentrations in unpreserved specimens taken for analysis during the first two or three days of storage.

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[1] Contribution No. 133 from the Woods Hole Oceanographic Institution. Contribution from the Bermuda Biological Station for Research, Inc.

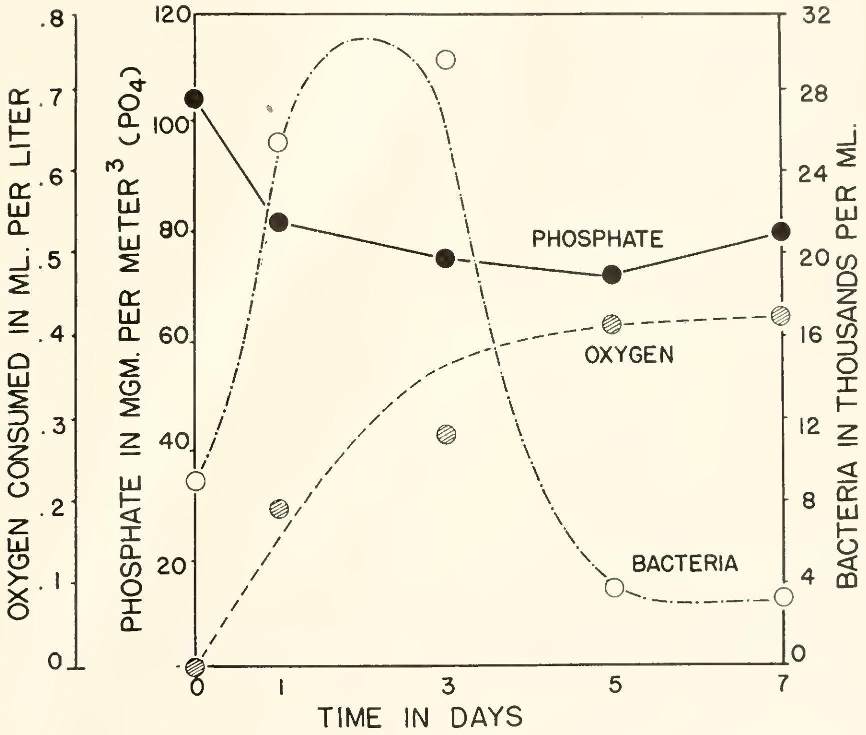
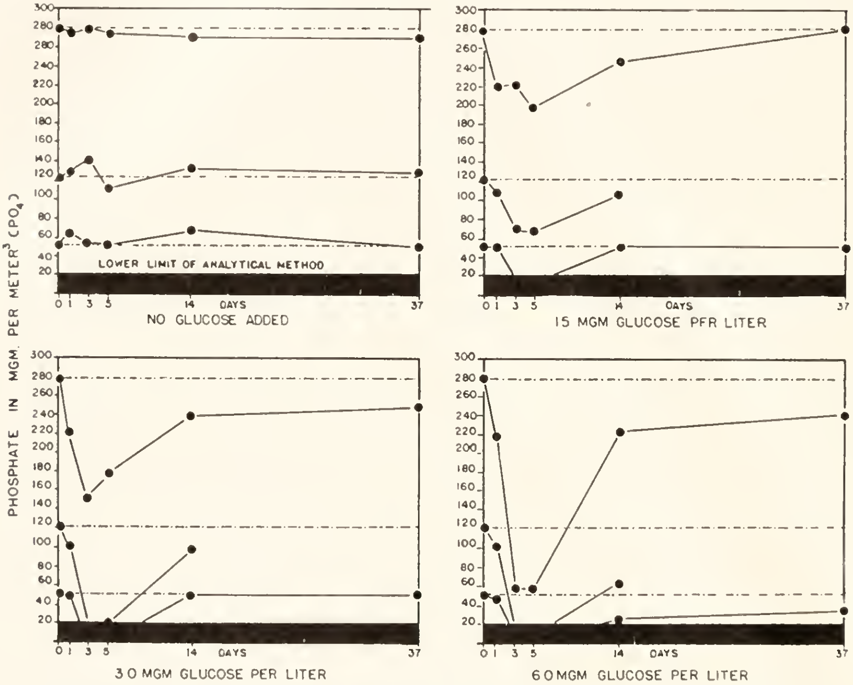


FIG. 1. Showing assimilation and regeneration of phosphate by bacteria in stored samples of sea water from Woods Hole Harbor—July 10, 1935. Phosphates in triplicate, other determinations in duplicate.

The first experiment was designed to relate these two behaviors—increase in bacterial numbers and assimilation of phosphate. Water from the mouth of Woods Hole Harbor was enriched with K_2HPO_4 to bring the phosphate content to about 100 mgm. per cubic meter, filtered through quartz sand, and dispensed into sterile, standard, oxygen demand bottles. These were stored in the dark at room temperature for varying lengths of time after which phosphate, bacterial number, and dissolved oxygen determinations were made. Figure 1, incorporating the results, clearly shows the rapid decrease in phosphate during the first three days of storage as noted before. Simultaneously the bacterial numbers and oxygen consumption reach their maximum. After three days the viable bacteria fall to a low level, and the oxygen uptake slackens off. On the seventh day there are signs of phosphate regeneration.



NO GLUCOSE ADDED

15 MGM GLUCOSE PER LITER

3.0 MGM GLUCOSE PER LITER

6.0 MGM GLUCOSE PER LITER

FIG. 2. Showing assimilation and regeneration of phosphate in stored sea water from off Bermuda, January, 1936. All determinations in triplicate.

There is, then, an interval of two or three days in which 20–25 mgm. of phosphate per cubic meter is bound as non-viable bacterial cells. (The count of viable cells has qualitative value only, indicating the phase of growth rather than actual numbers. Assimilated phosphate is held by a quantity of bacterial cells that is roughly proportional to the organic carbon oxidized during rapid growth—in this case an equivalent of 0.75 mgm. glucose per liter.) It has been pointed out that the length of time that phosphorus may be held by bacterial cells synthesized during decomposition of organic phosphorus compounds is significant in estimating their activity in phosphate regeneration. If this is long, then the process is retarded, but if, as this preliminary experiment suggests, the cells lyse within a short time, the turnover through their agency may be rapid.

In January, 1936, there was an opportunity to study the complete cycle of bacterial assimilation and regeneration under particularly favorable conditions, using the waters about Bermuda and the facilities at the Bermuda Station for Biological Research. The waters in this region are characteristically poor in phosphate, organic phosphorus, and available carbon—components that had proven troublesome in attempts to extend the experiments in Woods Hole. (A number of determinations showed the total phosphorus in shallow bays between St. Georges and Shore Hills to be less than 40 mgm. PO_4 per cubic meter.)

Sea water, taken from the surface about three miles southeast of Castle Roads, was filtered through quartz sand (imported), and enriched with varying amounts of phosphate and glucose, and a uniform excess of nitrate and iron. This was dispensed into a series of three-liter bottles and stored in the dark at room temperatures of 19° to 21° C. At intervals specimens were taken for phosphate analyses.

Results of the periodic inorganic phosphate analyses on the prepared, stored water are given in Fig. 2. Phosphate assimilation is complete in about five days, by which time it may be assumed bacterial growth has passed its maximum. Regeneration follows almost at once —rapid at first, and then leveling at fourteen days toward completion. Most of the phosphate is regenerated within this time, but a fraction, ranging from an eighth to a third, and greatest where relatively large quantities of glucose have been added, is still bound at the end of thirty-seven days when the experiment was terminated. It seems clear that bacterial cells do not bind phosphorus for more than a few days under storage conditions.

If autolyzing bacterial cells liberate phosphorus as phosphate there always exists the possibility that diatoms and other phytoplankton may similarly bring about direct regeneration. Two attempts to demonstrate direct regeneration gave negative results, but it will be recognized from the methods followed that the mechanism is by no means disproved. Ten-day cultures of *Nitzchia* grown in synthetic media were filtered and washed free of inorganic phosphate on a Seitz filter. The cells were suspended in 200 ml. of distilled water and washed on a second filter with 800 ml. of sea water, the lysate passing into a sterile aspirator bottle fitted with tubes for periodic sampling. Phosphate analyses at intervals of 14, 20, and 48 hours indicated no increase whatever in inorganic phosphate content.

A modification was arranged in which cultures of *Nitzchia* were washed on the Seitz filter, removed with a thin layer of the filter pad, lysed in distilled water for two hours, suspended in ten volumes of sea water, and allowed to stand. After three and eight hours, respectively, specimens were centrifuged and analyses made. These yielded no measurable amounts of phosphate.

Discussion and Summary

Bacteria, despite their potentialities as rapidly multiplying organisms, do not compete with higher phytoplankton for phosphate. Many factors may be suggested to account for their low numbers, but short life and brief viability period undoubtedly play a part. Under storage conditions where their rapid growth is favored they assimilate significant quantities of phosphorus, but this is quickly regenerated following their death. This suggests that the numbers of viable cells in natural water is not a complete index of bacterial activity, and that the efficiency of the organisms as agents of regeneration is inversely proportional to their life span.

The relations of the rapid growth phase, short period of maximum viable population, and the declining phase are not unique in marine bacterial cultures but are characteristic of bacterial populations in general (Buchanan and Fulmer, 1933, pp. 69–71). The curve representing them is the resultant of many growth and death curves over much shorter intervals, so that the number of cells actually produced during three, five, seven or fourteen days is indeterminately greater than the population at any one time.

Many other mechanisms for phosphate regeneration may be active in the sea. These experiments demonstrate one possible system, involving the agency of bacteria. They also offer an explanation for the insignificant amounts of bacterial cell substance in the nannoplankton.

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INDUCTION OF ENDOMIXIS IN PARAMECIUM AURELIA

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Attempts to induce endomixis in *Paramecium aurelia* have in the past led to conflicting results. Jollos (1916) and Young (1917) reported successful inductions; but Woodruff (1917) found that the time of occurrence of endomixis was but slightly modifiable by external conditions. Erdmann (1920) shared Woodruff's views on the inner determination of endomixis and rejected the results of Jollos and Young as unsound. The present paper, in general agreement with Jollos, reports a reliable method of inducing endomixis and sets forth the quantitative differences in response to this method at different periods of the interendomictic interval and in different stocks of the species.

The procedure employed for inducing endomixis was that which has been described in earlier papers (Sonneborn, 1936). The organisms were under culture in isolation lines under uniform conditions. To induce endomixis, the surplus animals available after the daily transfer of the lines to fresh culture fluid were collected into 10 drops (approximately 625 cu. mm.) of fresh culture medium in a Columbia dish, and were kept without further change at 31° ± 1° C. By culture in this manner endomixis was induced, as hereafter described.

Two stocks of *Paramecium aurelia* were studied with relation to the induction of endomixis: these were the stock R, dealt with in earlier papers by the author and associates, and Woodruff's long-lived stock, here designated W. At various times over a period of three years, 11 groups of lines of stock R and 8 groups of stock W were investigated.

Of the 19 groups studied, 18 were started with one or a few animals in the climax of endomixis, while one was started with 24 exconjugants. In each group begun with animals in endomixis, the few original animals were soon expanded into usually 12 isolation lines. In all groups, one or more individuals from each isolation line were stained daily, to determine the occurrence of endomixis. Any line that was found in endomixis was discarded, in order that the interval of time since the previous endomixis should be the same for all the individuals placed in any one of the small mass cultures.

The mass cultures derived as above described from these isolation lines were observed at intervals not exceeding 12 hours, to determine whether conjugation was in progress. Also, a random sample of 20 to 50 individuals of each culture was stained usually once a day to determine whether endomixis was in progress.

Stock R

As set forth in an earlier paper (Sonneborn, 1936), mass cultures of stock R set up soon after endomixis or conjugation, in the way above described, soon pass into conjugation, so that such cultures are unavailable for the study of the induction of endomixis. But when the interval from the preceding nuclear reorganization (endomixis or conjugation) is greater, conjugation does not occur, but some of the individuals pass into endomixis. The percentage of those thus undergoing endomixis was accurately determined, and compared with the percentage occurring in the isolation cultures (still in progress) from which they were derived. In this way the effect of the mass culture conditions in inducing endomixis is numerically expressed.

Table I

Stock R. The percentages of daily isolation lines at 27.5° C. in endomixis on successive days of the interendomictic interval, compared with the percentages of animals in endomixis in mass cultures at 31° C. on corresponding days. In each case, the mass cultures had been derived from the isolation lines on the preceding day.

Day since initial endomixis	Percentage of endomixis	
	In isolation lines at 27.5° C.	In mass cultures at 31° C.
9	0	0
10	0	0
11	0	0
12	8.3	37.6
13	0	58.7
14	0	39.7
15	16.7	53.0
16	42.9	93.4
17	0	98.3
18	50.0	97.5
19	57.1	97.5
20	0	100
21	66.7	97.0
22	0	100
23	0	100
24	100	100

Typical results are given in Table I. From the twelfth until the twenty-fourth day after the previous endomixis, the percentage of individuals in endomixis in the mass cultures is always much greater than that in the isolation cultures (until by the twenty-fourth day the last isolation line has passed into endomixis, bringing the comparison to a close). With increasing time since the preceding endomixis, the percentage of individuals in which endomixis was induced quickly rose from 37.6 per cent on the twelfth day to 98.3 per cent on the seventeenth day, after which it remained at or near 100 per cent. Similar results, differing only in details, were obtained with the other groups of stock R, including the group begun with ex-conjugants.

These results thus confirm the reports of Jollos (1916). Endomixis is induced by the treatment set forth and, within the range of intervals examined, it is induced less readily the shorter the interval since the preceding endomixis. This relation to the time since a previous endomixis is also shown in other ways. (1) It took a longer period in mass culture to induce endomixis in the earlier mass cultures derived from given isolation lines than in the later ones. Thus the mass cultures set up on the seventh to tenth days of a certain series yielded no endomictic animals until these cultures were two days old, whereas in the later cultures of the series endomixis was induced in one day. (2) The production of large percentages of endomixis in the early cultures required a type of treatment that was unnecessary in the later cultures. Thus, a culture set up on the tenth day after endomixis yielded no more than 10 per cent endomixis on any of the four days it was examined, but a subculture from it set up on the third day and provided with fresh culture fluid gave 44.4 per cent endomixis on the following day. Similar results were obtained with other cultures: a culture set up on the eleventh day after endomixis gave but 5.4 per cent endomixis on the second day; it was then divided into two parts one of which was provided with 5 drops of fresh culture fluid, the other not. On the next day, the former had 69.2 per cent endomixis, the latter 5.8 per cent. It appeared that the percentage of endomixis in these early cultures reached its maximum at about the time when the food supply was giving out and that further increase in the percentage of endomixis could be obtained only by supplying additional food.

The relations observed were usually of the type just set forth, but very rarely exceptional results were obtained. In lines descended from one endomictic individual, mass cultures set up on the first, third, and fifth days after endomixis yielded at once another endomixis instead of conjugation, as would normally have been expected at this period. These lines also went into endomixis after 5 to 8 days in the isolation cultures. It is perhaps significant that in this case the initial nuclear reorganization was highly abnormal: on the day following the stage of complete macronuclear fragmentation there were neither numerous small fragments nor pale anlagen of the new macronucleus, such as normally occur; instead, several large, aggregated spheres of chromatin were present. Thereafter, normal-appearing macronuclei were characteristic of this line. Similar abnormal reorganizations have on several occasions been followed by extraordinarily short intervals, and appear to be the basis of the unusually quick recurrence of endomixis in these cases.

Stock W [1]

In stock W, Sonneborn and Cohen (1936) have shown that mass cultures set up in the way here set forth do not yield conjugants, and examination at not more than 12-hour intervals showed that conjugation did not occur in the present cultures. This made it possible to determine from the start the percentages of individuals in endomixis.

In Table II are given, for a typical group of lines of stock W, the percentages of individuals in endomixis in the mass cultures set up on successive days of the interendomictic interval and kept for various periods of time at 31° C. The table is to be read as follows: the culture set up on the second day yielded no endomixis; the culture set up on the third day yielded 8.6 per cent endomixis after five days at 31° C., i.e., $5 + 3 = 8$ days after the initial endomixis and so on. Table II shows that endomixis was regularly induced every day beyond the seventh after the initial endomixis. In the isolation lines endomixis did not occur until the thirtieth day.

In some of the other groups, the earliest induction of endomixis occurred sooner than in the typical group. In two groups endomixis was induced four days after the initial endomixis and in one group, three days. Usually, however, at first only small percentages of endomixis could be induced after 5 days of treatment. Later, comparable small percentages were induced in four days of treatment. Still later, treatments of four days yielded nearly 100 per cent endomixis; then 100 per cent endomixis was obtained in three days and finally in two days. In contrast to stock R, which yielded 100 per cent endomixis in one day in the later cultures of a series, stock W never yielded 100 per cent endomixis in less than two days of treatment.

In stock W, as in stock R, the percentage of induced endomixis increased with the time since the preceding endomixis. This is shown in Table II and also by a comparison of the percentages of endomixis induced on the same day in the cultures of several groups differing in time since the last endomixis. Thus, the mass cultures set up on a certain day yielded after two days 100 per cent in a group 24 days past endomixis, 97.4 per cent in a group 19 days past endomixis, and 8.2 per cent in a group 4 days past endomixis. In two-day-old cultures set up on another day there were 100 per cent in a group 23 days past endomixis, 30.8 per cent in a group 18 days past endomixis, and 0 per cent in a group 13 days past endomixis.

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[1] In the work with stock W, the author was greatly assisted by Dr. B. M. Cohen.

Table II

Stock W. The percentage of individuals in endomixis in mass cultures set up daily from a group of isolation lines. Cultures set up from the first to the twenty-eighth day after the initial endomixis and kept at 31° C. for 1 to 5 days. During the entire period there was no endomixis in the isolation lines from which the mass cultures were derived.

Days from endomixis till mass cultures set up	Percentage of endomixis in mass cultures				
	Number of days since culture set up				
	1	2	3	4	5
2	0	0	0	0	—
3	—	—	—	—	8.6
4	—	—	—	—	29.7
5	—	—	—	6.5	—
6	—	—	—	—	26.8
7	—	—	—	83.9	—
8	—	—	65.6	97.1	—
9	—	32.3	69.1	97.8	—
10	0	36.7	100	—	—
11	0	96.9	—	—	—
12	0	56.0	100	—	—
13	0	60.6	100	—	—
14	0	100	—	—	—
15	3.3	100	—	—	—
16	0	96.3	—	—	—
17	20.0	97.4	—	—	—
18	0	—	100	—	—
19	—	92.9	—	—	—
20	0	100	—	—	—
21	—	100	—	—	—
22	10.5	100	—	—	—
23	—	85.3	—	—	—
24	—	93.3	—	—	—
25	—	48.4	47.6	48.5	50.0
26	—	23.5	77.8	—	—
27	—	88.9	—	—	—
28	—	96.9	—	—	—

On the other hand, factors other than the time since the last nuclear reorganization influence the percentage of endomixis induced. There were many instances in which the percentage of endomixis was less at a later period after endomixis than earlier. For example, in Table II, there was 96.9 per cent endomixis after two days treatment of the culture set up on the eleventh day after endomixis, and only 56.0 per cent in the culture set up a day later. Such irregularities were probably due to differences in the environmental conditions at different times, as indicated by the fact that the same departure from the general trend was usually shown by all the cultures set up on a particular day. An example of the effect of one such environmental change was provided by the accidental rise of temperature to 35° C. in the box ordinarily kept at 31° C. In four groups of cultures exposed to this high temperature, the percentages of endomixis were 25.7 per cent, 32.9 per cent, 36.0 per cent, and 0 per cent, as compared with 79.8 per cent, 75.1 per cent, 96.7 per cent, and 43.9 per cent, respectively, for these same four groups on the two preceding days at 31° C. Thus, rise of temperature from 31° to 35° reduced the percentage of endomixis induced; although, as earlier shown, rise from 27.5° to 31° greatly increased the percentage of endomixis. There are doubtless other as yet unanalysed factors influencing the induction of endomixis.

Summary

In both stocks R and W of *P. aurelia*, endomixis can be induced by placing at 31° C. small mass cultures containing the surplus animals from isolation lines. In stock R, induction cultures set up soon after endomixis yielded conjugants, so that the induction of endomixis could not be studied quantitatively in these. Later cultures, however, showed increasing percentages of induction until 100 per cent was obtained after one day at 31° C. Earlier cultures which gave but low percentages of endomixis with this treatment could be induced to give larger percentages by subculturing or adding some fresh culture medium to the original induction culture. In a few exceptional cases, abnormal reorganization of the nuclear apparatus was soon followed by a normal endomictic reorganization in isolation lines, and by the induction of endomixis instead of conjugation in the induction cultures set up immediately after the abnormal reorganization.

In stock W, the absence of conjugation made it possible to examine the percentages of endomixis induced at all periods of the interendomictic interval. During the first few days after an endomixis, it required prolonged treatment to induce even a low percentage of endomixis, but as the time since the last endomixis increased, higher and higher proportions of endomixis were induced with shorter and shorter treatments, until 100 per cent endomixis could be induced in two days. Irregularities in this progression are probably due to environmental differences in treatment from day to day. Rise of temperature above 31° C. was shown to reduce the percentage of endomixis induced. Other conditions also probably play a similar rôle.

Stocks R and W differed in two respects in regard to their reaction to the conditions favoring the occurrence of endomixis. (1) In stock W, endomixis could regularly be induced 7 days after a preceding endomixis, and in some cases, as early as the third day; but in stock R, the typical response at this period was conjugation, not endomixis. (2) Late in the series of induction cultures, 100 per cent endomixis could be induced with a treatment of one day in stock R, but treatment of two days was required in stock W.

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SOME EFFECTS OF DIET ON THE GASTRIC EPITHELIAL CELLS OF THE GRASSHOPPER, MELANOPLUS DIFFERENTIALIS THOMAS[1]

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Introduction

The effects of variation in diet on various tissues has been investigated in many species of vertebrates (d'Ancona, 1927; Truszkowski, 1927 and 1928; Tirelli, 1928; Smith, 1929; Kremer, 1932). The aim of this study is to determine the effect of diet on the tissues of the grasshopper. A preliminary study disclosed that the epithelial cells of the anterior lobes of the gastric cæca are most favorable for this study.

The six gastric cæca in this species are arranged in a circle around the cephalic end of the mid gut as bilobed diverticula. which are thicker in the anterior lobe than in the posterior. The gastric epithelium is thrown into 6 large and 6 to 8 smaller, inwardly projecting longitudinal folds or villi which are the most concerned with secretion (Woodruff, 1933).[2] Between these villi are longitudinal crypts corresponding in number and arrangement with the villi. This epithelium is composed of functional secretory cells which are elongate and columnar, and are provided distally with a thick striated border of cilia-like filaments. Scattered irregularly through the epithelium are the usual nidi of regenerative cells for replacement.

Materials and Methods

The grasshoppers were reared on the leaves of barley or wheat, alone or in combination, and on oat leaves alone. It had been previously determined from growth data (Hodge, 1933) that any of the above diets except oat leaves are satisfactory. This study also shows that the various satisfactory diets do not differ materially from each other in their effect on the cells.

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[1] Contribution No. 8 from the Biological Laboratories of Temple University.

[2] "'Villus,' as used by Sanford (1918) is preferable to 'fold,' as applied to the typical elevations in the gastric cæca, inasmuch as these elevations are permanent structures. Even 'villus' is not entirely satisfactory for the reason that the structure is so extensive in a longitudinal direction," Woodruff (1933), p. 56.

Alimentary tracts from the grasshoppers so reared were fixed in B3 (Carother's modified Bouin), sectioned at 12 microns, and stained with hæmatoxylin and eosin. Eleven cæca of satisfactorily fed grasshoppers and nineteen of those fed on oat leaves were studied. The diameter of the cæcum was measured in four directions and an average taken. The number of cells per section, the number of secretory inclusions, and the number of mitoses visible in the nidi were counted.

One hundred cells located on the villi, 100 from the crypts of the satisfactory diet, 147 from the villi and 148 from the crypts on the oat diet were studied. Usually 5 and never more than 10 cells from any one section were selected. The length and width of the cell and nucleus and the length of the filaments of the striated border were measured.

The volume of the cells was calculated as a rectangular prism. In areas where some cells were sectioned transversely, the cells varied in number of sides from 3 to 6. The majority had 4 sides and the remainder varied in approximately equal numbers on either side of that figure. The volume of the nuclei was calculated as an oblate spheroid.

Results

Cæcum Diameter

The gross morphology of the cæca and the pattern of distribution of the histological elements were not affected by diet. As previously determined (Hodge, 1933), growth on the oat diet is slow, irregular, and retarded, and mortality is high. Only one adult was available for this study. It showed an average diameter through the thickest part of the cæcum of 1,173 microns. Others in the last instar ranged from 667 to 992 microns, with the average 791. The average for all on the oat diet including the adult was 890 microns. On the satisfactory diet all were adults. On this diet the cæcal diameter ranged from 670 to 1,169 microns, with an average of 952. Thus the adults on the satisfactory diet showed an average cæcal diameter 1.07 times that of the 18 last instar and one adult individual on the oat diet; or the oat diet, 93.5 per cent that of the satisfactory. In view of the wide individual variation, this figure is hardly significant. Gross morphology is not affected by this difference in diet.

Number of Cells

The number of cells per section on the satisfactory diet ranged from 365 to 775 (average 552.7). The number on the oat diet, from 340 to 648 (average 489.0). Thus the average number of cells per section for the satisfactory diet was 1.13 times that for the oat diet; or the oat diet, 88.5 per cent that of the satisfactory.

A comparison of the number of cells per unit area is difficult. No two cæca are exactly the same shape nor are they provided with villi and crypts to exactly the same extent. However, a ratio between the average diameter of the cæcum and the number of cells yields a rough comparative figure. For the satisfactory diet this ratio ranges from 1.34 to 2.38 for the various cæca, with an average of 1.79. For the oat diet the range is 1.36 to 2.02 with the average 1.82. Thus the average ratio for the satisfactory diet is 98.4 per cent that of the oat diet; for the oat diet, 1.02 times that for the satisfactory.

TABLE I

	Inclusions per Section		Cells per Section	Percentage of Cells with Inclusions			Ratio, Satisfactory: Oat Diet			Ratio, Oat: Satisfactory Diet		
	Granules	Vacuoles		Granules	Vacuoles	Either	Granules	Vacuoles	Both	Granules	Vacuoles	Both
Satisfactory diet												
Minimum	12	29										
Average	35.8	43.5	552.7	6.5	7.9	14.3	1.35	2.93	1.86			
Maximum	83	78										
Oat diet												
Minimum	12	8										
Average	23.5	13.2	489.0	4.8	2.7	7.7				.738	.342	.538
Maximum	30	30										

Rate of Replacement of Cells

Characteristic of insect mid-gut epithelium are the nidi from which new secreting cells for replacement proliferate by mitosis. The number of mitoses visible at any one time gives an indication of the rate of replacement. The satisfactory diet showed 4 to 8 mitoses per section, with an average of 6.4. The oat diet showed 8 to 14 with an average of 11.6. Thus the rate of replacement for the satisfactory diet was 55.2 per cent that of the oat diet; or the oat diet, 1.8 times that for the satisfactory.

Secretory Products

By the technique used here the secreting epithelial cells show two types of inclusions: darkly-staining granules and clear vacuoles. They originate just distad to the tip of the nucleus and are progressively larger as they are found successively nearer the distal end of the cell. I interpret them as some of the gastric secretory products (Hodge, 1936). The numbers of these inclusions are given in Table I. Thus the satisfactory diet showed 1.35 times as many granules, 2.93 times as many vacuoles, or 1.86 times as many inclusions, as the oat diet. The oat diet showed 74 per cent, 34 per cent, and 54 per cent, respectively, as many as the satisfactory.

Dimensions of Cells

The average values in microns and cubic microns for the dimensions measured: length, width and volume of cell and nucleus, and width of striated border, are tabulated below. The average for all the cells on the satisfactory diet differed only very slightly from that for all the cells on the oat diet. The greatest variation is seen in nuclear volume and the length of the filaments of the striated border. In general the cells and their nuclei on the satisfactory diet are slightly longer and more slender than those on the oat diet and the filaments of the striated border are shorter:

Diet	Cell Length	Cell Width	Cell Volume	Nuclear Length	Nuclear Width	Nuclear Volume	Striated Border	Nucleo-plasmic Ratio
Satisfactory...	61.6	12.2	9484.7	16.9	8.1	580.7	5.0	1 : 16.3
Oat..........	58.3	12.6	9450.7	16.3	8.6	663.0	5.6	1 : 14.3

In view of the great variation between minimum and maximum values for these various dimensions (see the more extended data in Table II) these differences are hardly significant. More striking differences are observed in comparing the dimensions of cells located on the villus and crypt areas respectively. On the satisfactory diet the cells vary greatly in relation to their location on these two areas. In general on the villi the cells are shorter and wider, the striated borders narrower, and the nuclei more slender than in the crypts. On the oat diet the cells of the two areas are more similar. This may be summarised as in Table II.

Discussion

The diet of oat leaves alone is undoubtedly unsatisfactory for this species. This has been shown by rate of growth (Hodge, 1933) and by abnormalities in cytology of the germ cells (C. E. McClung, personal communication, unpublished).

This study shows that the rate of replacement of the cæcal epithelial cells on the oat diet is almost double that on the satisfactory. Replacement is more or less continuous in insect mid-gut epithelium. Apparently it is necessitated by the exhaustion of holocrine secretory cells, the gradual wearing out of merocrine cells, or other factors.

Table II

	Satisfactory Diet			Oat Diet		
	Villus	Crypt	Ratio Villus : Crypt	Villus	Crypt	Ratio Villus : Crypt
Cell length						
Minimum..........	29	44	.8 : 1	40	37	.9 : 1
Average...........	55.9	67.0	.8 : 1	56.7	59.8	.9 : 1
Maximum.........	95	99		88.1	84	
Cell width						
Minimum..........	6	8		6	6	
Average...........	12.4	12.0	1.0 : 1	13.2	12.0	1.1 : 1
Maximum..........	18	17		21	18	
Cell volume						
Minimum..........	2245	3213		2069	2829	
Average...........	8891.4	10077.9	.9 : 1	10012.7	8902.3	1.1 : 1
Maximum..........	26741	22289		23286	23621	
Nuclear length						
Minimum..........	11	11		9	11	
Average...........	16.9	16.9	1.0 : 1	16.6	16.0	1.0 : 1
Maximum.........	23	26		26	22	
Nuclear width						
Minimum..........	5	6		5	5	
Average...........	6.7	9.0	.7 : 1	8.1	9.1	.9 : 1
Maximum.........	11	14		13	16	
Nuclear volume						
Minimum..........	171.5	367.6		111.9	252.2	
Average...........	364.4	796.9	.5 : 1	561.9	762.0	.7 : 1
Maximum..........	838.3	2060.5		1372.2	1658.9	
Striated border						
Minimum..........	2	3		3	4	
Average...........	3.7	6.3	.6 : 1	4.9	6.4	.8 : 1
Maximum..........	6	12		8	11	
Nucleo-plasmic ratio....	1 : 24.4	1 : 12.6	.5 : 1	1 : 17.8	1 : 11.7	.7 : 1

On the oat diet the number of the large holocrine type of secretory inclusions demonstrated by the technique used here is less than on the satisfactory diet. If the greater replacement results from overstrenuous secretion, it must be of the merocrine type demonstrated by Woodruff (1933) and others.

On the satisfactory diet the more actively secreting cells of the villi are conspicuously smaller than the crypt cells. From this one is led to the generalization that among these cells the smaller type is the secreting type. On the oat diet the cells, though shorter in length of nucleus and cell body, are yet for all other dimensions as large or larger than those from the satisfactory diet, and in each case show less difference between the villus and crypt areas.

On the whole, therefore, the villus and crypt cells on the oat diet are more similar morphologically than the villus and crypt cells on the satisfactory diet. Furthermore, their general morphology approaches more nearly to that of the larger, less actively secreting crypt cells. If morphology be taken as a criterion of secretory function the oat cells are not secreting more strenuously, but less.

Other factors that suggest themselves as reasons for wear and replacement are the results of toxic influences, and inanition. Toxic materials resulting from the digestion of the oat leaves have not been demonstrated directly, but the high mortality among grasshoppers on the oat diet (Hodge, 1933) may be due to toxicity. The fact that the cells on the oat diet are provided with longer filaments of the striated border is also suggestive. Longer filaments might conceivably have a distinct protective function, especially since these can be demonstrated to be covered with a mucous layer in the living condition (Hodge, 1936).

Inanition in other material (Miller, 1927, on rats; Sun, 1927, on mice) produces loss of digestive epithelium. This desquamation, in animals so dissimilar on the part of a tissue not normally so adapted, cannot be too closely analogized to the replacement in the grasshopper. But it may well be that the greater replacement on the oat diet may be due to at least two factors—toxic effect of the diet and a partial inanition.

The two types of large secretory inclusions demonstrated by the technique used here—namely, granules and vacuoles—are shown in noticeably larger number on the satisfactory diet than on the oat diet. One and one-third times as many granules, and almost three times as many vacuoles were counted on the satisfactory diet as on the oat diet.

The nature and function of these two types of inclusions are as yet uncertain. If they represent two distinct types of enzyme material, it would seem that the oat diet hinders the formation of the vacuolar type more than that of the granular type. However, in each case either some dietary component necessary for their elaboration is relatively deficient in the nutrient materials the grasshopper can obtain from the oat leaves, or an injurious material either directly or indirectly obtained from the same source inhibits their elaboration. This difference may be the result of a generalized partial inanition, but in other material inanition leads to vacuolization rather than the reverse (d'Ancona, 1927; Tirelli, 1928; Miller, 1927).

The difference in dimensions between the crypt and villus cells on the satisfactory diet is understandable from the fact that the villi are the more actively secretory (Woodruff, 1933). In the case of each dimension studied except cell width and nuclear length the cells of the villi give much the smaller figure. The corresponding dimensions for the oat diet, while in most cases smaller for the villus than for the crypt cells, do not show such wide divergence. For some dimensions, in fact, the average value for the villus cells is greater than for the crypt cells. Thus on the oat diet the villus cells in size are more like the crypt cells which secrete less; and from this morphological criterion are less differentiated for the normal function of villus cells,—namely, secretion.

The slightly higher nucleo-plasmic ratio on the oat diet suggests an inanition. Such an increased nucleo-plasmic ratio has been reported in other material during inanition (d'Ancona, 1927, on eels; Kremer, 1932, on frogs; Truszkowski, 1927, on dogs, and 1928, on frogs).

A comparison of the relation of the nucleo-plasmic ratio in the crypt areas with that in the villi shows that on the satisfactory diet the ratio in the crypts is twice that in the villi, while on the oat diet it is only 1.5 times as great. This suggests again the conclusion borne out by the dimensions of the cells, that there is less difference between the types of activities of the cells in these two areas respectively on the oat diet than on the satisfactory.

The difference in length of the filaments of the striated border is less readily explicable. The exact purpose of the striated border is not yet well understood. The secretory products are discharged through this border. This is effected by total rupture of the border or by a temporary separation of the filaments into clumps or pencils, depending on the nature of the secretory operation at the moment. The shortness of the filaments on the actively secreting villi on the satisfactory diet would seem to be an adaptation to this function, to permit easy passage of the secretion.

If the striated border subserves any active part in secretion it would seem from the length on villus and crypt respectively on the satisfactory diet that shortness of filaments is in some way correlated with active secretion. From this point of view the greater length of the filaments on the oat diet, correlated with the lesser number of inclusions, points to decreased secretion on this diet.

The fact that on the oat diet the filaments on the villi are more nearly the length of those in the crypts suggests again a partial relinquishment of the active secretory function of the villus. This area seems to have assumed a more generalized condition more nearly resembling the crypts.

If the striated border serves more particularly as a protective surface, the increased length of the filaments on the oat diet would point to a greater need for such protection. Some product of injurious nature from the wholly or partially digested oat leaves is indicated. The shorter filaments shown on the satisfactory diet would indicate that such products are not liberated in the digestion of the wheat or other satisfactory food plants.

At least three factors are indicated by this study as contributing to the various effects of the unsatisfactory oat diet. Inanition is evidenced by the greater rate of replacement and nucleo-plasmic ratio on the oat diet. The retarded growth observed in rearing the animals (Hodge, 1933) is further evidence of this. Injury by or toxicity of some product of digestion or component of the oat leaves is also suggested by the increased rate of replacement. Perhaps the longer filaments of the striated border are also a response to some such factor, as a protective adaptation. The mortality observed in rearing the insects (Hodge, 1933) points toward this as well. Loss of secretory ability on the part of the cells on the oat diet, and especially in the villi, is evidenced by the lesser number of secretory inclusions, as well as by the approximation in dimensions of the cells of the villi to a size nearer that of the cells of the crypts.

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LICNOPHORA LYNGBYCOLA, A NEW SPECIES OF INFUSORIAN FROM WOODS HOLE

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The genus *Licnophora* (Infusoria Heterotricha) includes some well characterized marine species; Kahl's revision of the group (1932 and 1933) is a convenient guide in their identification.

I have observed a species which does not seem to have been described yet. As a matter of fact, there is but little difference between it and the species already known in the morphological characters, but, in a biological sense, this new species is quite peculiar in its constant association with an alga from the group of the Cyanophycea, which it uses as a support.

I first found this species at Woods Hole in July, 1929, and found it again at Concarneau in July, 1936. In both cases, the cultures of this infusorian and of the alga on which it clings had grown on glass-plates, immersed in running sea water, either in the aquaria of the Marine Biological Laboratory at Woods Hole, U. S. A., or in the Laboratory's fish-ponds at Concarneau, France.

I shall describe this species under the name of *Licnophora lyngbycola,* n. sp.

Structure

The infusorian is 100 μ long, and equally wide. The general form is changeable, often globular. The peristome is very largely everted in the fully expanded infusorian. In this case the ventral side is limited by an anterior brim, nearly circular, slightly depressed, and limited itself by the adoral zone.

The protoplasmic mass constitutes a dorsal rounded lump, more or less prominent and more or less wrinkled. The body becomes narrower towards the lower part, taking the shape of a straight foot, only 20 to 25 μ wide, which then becomes wider once more, forming a cup-like organ of fixation.

The mouth is placed ventrally, in the inferior third part of the body, under the protection of a thin protoplasmic lip. The adoral zone, at first horizontal in the whole of its pharyngeal part, rises in a circular curve along the inward left side of the peristome, runs along the upper edge, comes down again on the right side, and finally, by means of a more pronounced curve perpendicular to the axis of the body, it follows a sudden protoplasmic ventral prominence which stands off in front of the mouth, and stops short at the point where this prominence comes down towards the cytostome, stretching out the slender cytoplasmic lip, already mentioned.

The fixation apparatus consists of a kind of circular cup, almost 20 μ wide, sustained and edged by a skeleton ring, refracting and homogeneous; this ring, which is bent saddle-like, shows the most appropriate form for the fixation of the animal on the alga. Around it all there is a pedicular fringe, surmounted by a velum, that is to say, a slender and circular fold of the protoplasmic wall of the body.

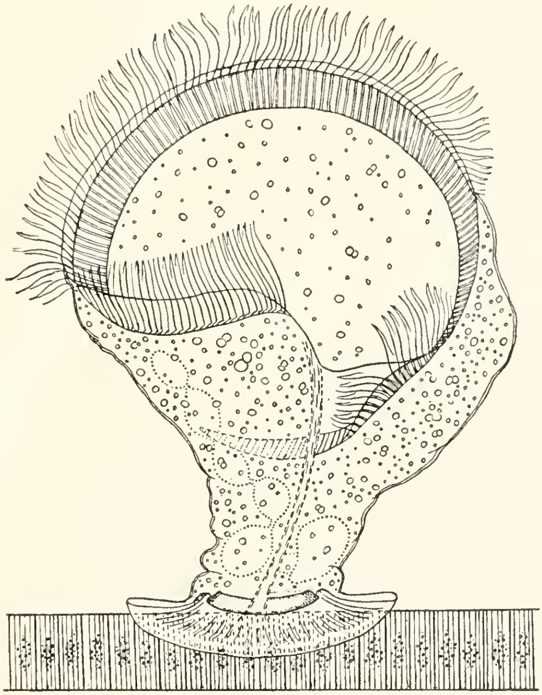

FIG. 1. *Lycnophora lyngbycola,* attached on the body of the Cyanophycea.

A refracting, contractile fiber is fixed on the ventral side of the attaching disc and on the terminal edge of the adoral fringe, passing in front of the pharyngeal opening.

The cytoplasm is normally of the thready type, filled up with food in the gastric vacuoles at different states of digestion, heaped up in the dorsal lump.

The macronucleus is beaded; it is made up of three rather large segments displayed in the manner of a horseshoe over the attaching disc, and of a series of approximately 8 or 10 smaller segments, which compose a semicircular row of beads, going upwards on the right side and stretching dorsally along the right and superior sides of the adoral zone. A spherical and single micronucleus is situated under the pedical segment of the macronucleus.

The protoplasmic body is enclosed by a rather rigid pellicle, with double outline, slightly and closely punctuated, more or less wrinkled,

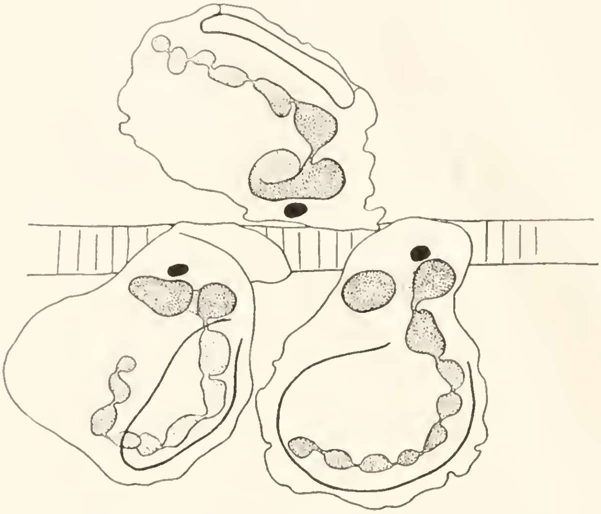

FIG. 2. Schema of three individuals, showing the beaded macronucleus (dotted) and the micronucleus (black).

and united by a protoplasmic net-work to the principal cytoplasmic mass. The infusorians of Woods Hole showed some polygonal plates in their pellicle, with joint-lines; but at Concarneau they were only stippled.

These infusorian individuals are found on the filaments of a colorless Cyanophycea which belongs probably to the group of the Lyngbyæ. These threads, fixed by one end on a solid support, stand up almost vertically, bearing the infusorians, sometimes one only, sometimes a group of ten or twelve individuals. As they change places nearly all the time, one can watch them as they move upwards and downwards, pushing one another. In making these movements, they bend their peristomes in all directions, and in normal conditions they will move all

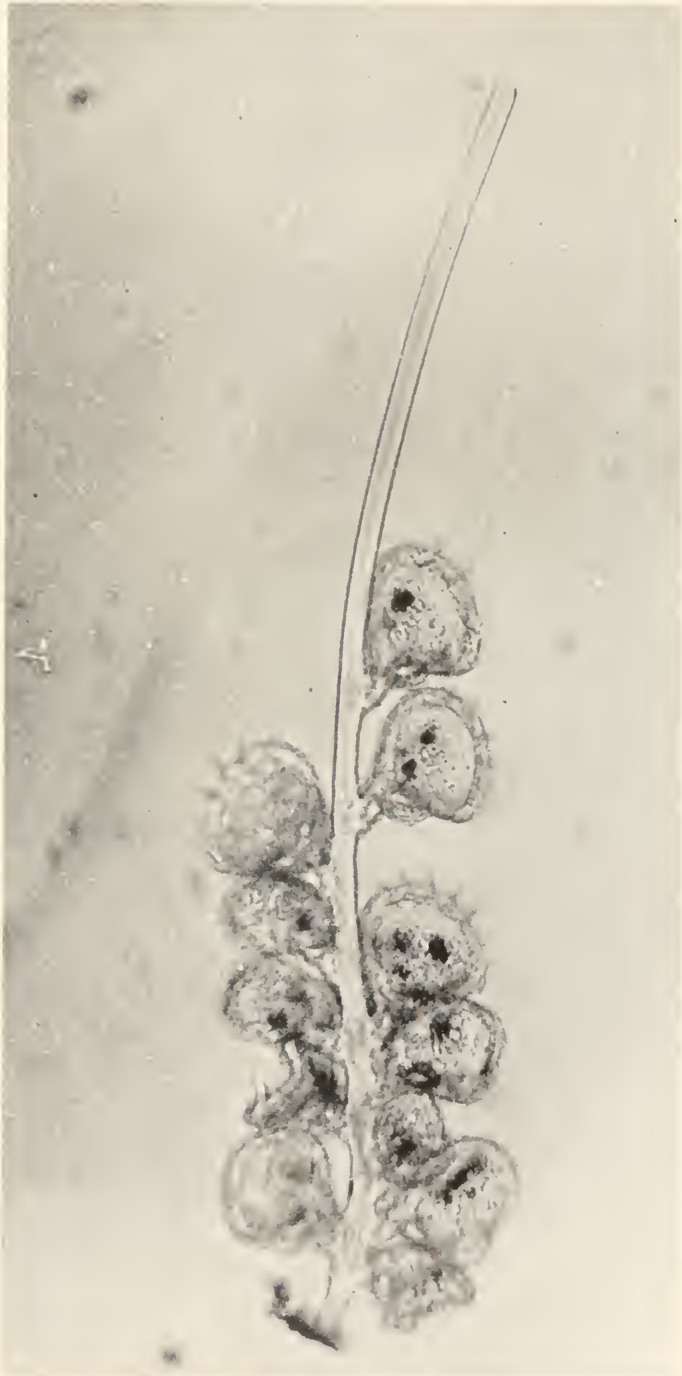

FIG. 3, *a* and *b*. Microphotographs of a group of *Lycnophora lyngbycola* on the Cyanophycea, in living state.

around the alga, without showing the slightest tendency to leave it. Now if, by a manipulation, you detach them mechanically, they begin to swim freely, but it seems that they are incapable of fixing themselves on any plane or even irregular surface, the attaching disc always maintaining its rigid and bent shape; this shows clearly the very close and typical adaptation of this infusorian to the Cyanophycea which bears it.

At first glance, a group of *Licnophora lyngbycola,* seen under low power, looks like a colony of *Epistylis.*

In another way, the general appearance of these infusorians, when in groups, fixed as they are on their rigid thread as if on a stalk, might be compared with a form found in the vegetable kingdom, the rose-mallow, *Althea* inflorescence.

Licnophora lyngbycola, n. sp., is a form rather close to *L. Kohni,* Clap. et Lach., considering the structure of the peristome, but it stands apart in regard to its very prominent dorsal lump, the bent cup of fixation, the shape of the macronucleus, and the very close adaptation to the vegetal support on which it lives.[1]

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[1] For bibliography, refer to: Kahl, A., 1932. Wimpertiere oder Ciliata, in *Die Tierwelt Deutschlands.* G. Fischer, Jena; Kahl, A., 1933. Ciliata libera et ectocommensalia, in *Die Tierwelt der Nord- und Ostsee.* Leipzig.

SOME EFFECTS OF NUMBERS PRESENT ON THE RATE OF CLEAVAGE AND EARLY DEVELOPMENT IN ARBACIA

W. C. ALLEE AND GERTRUDE EVANS

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Frank and Kurepina (1930) reported accelerated development for sea urchin eggs, species not given, when 10 to 20 eggs were present per drop as compared with the rate when only one or two eggs were present. They used hanging-drop cultures and determined the relative degree of development at varying times after fertilization. Apparently they did not examine early cleavages. They interpreted their findings as being the result of the action of mitogenetic rays. Allee and Dr. J. R. Fowler began the exploration of this problem at Woods Hole during the summer of 1931 and obtained preliminary results for *Arbacia punctulata* which indicated that fertilized eggs left standing in drops of varying size for 16 hours or longer showed a decided tendency toward faster development in the more crowded as compared with the less crowded populations. At times, however, isolated eggs would develop as rapidly and as perfectly as did those in the somewhat crowded cultures. In these preliminary and hurried experiments there was no steady difference in the time to 50 per cent cleavage for the first two cleavages which were the only ones studied.

The present authors took up this problem in the summer of 1934. The methods of handling the sea urchins, of procuring eggs and sperm and of fertilization were based closely on the directions given by Just (1928). Usually freshly collected animals were used one to two days after being delivered in our laboratory. Later in the season eggs and sperm were obtained from sea urchins which had been collected for some time and, by being kept in the laboratory, still retained their sexual products when those collected daily were mostly spent.

Specifically we dried the animals on paper towels, meantime pressing the spines out of the way and breaking several loose from the body. The urchins were then opened by cutting with scissors around the peristome; Aristotle's lantern was removed with tweezers and the edge of the peristome shell was broken somewhat. The body fluid was shaken out and the animals thus prepared were placed aboral side down in a clean, dry Syracuse watch-glass. Normally eggs were soon shed by one or more of the females. These were removed after extrusion by pipetting through washed new cheese cloth into finger-bowls containing filtered sea water and were decanted through several changes of similar sea water. Shed sperm were selected according to Just's criteria, covered and left until needed. One drop of the "dry" sperm was diluted in 10 cc. of filtered sea water in a Syracuse watch-glass. At first this was used almost immediately but beginning with the hanging-drop experiments diluted sperm was allowed to stand for 2 to 3 minutes before being used to inseminate eggs. Fertilization was accomplished by adding two (or sometimes one, or three) drops of this suspension to the eggs in 250 cc. of sea water in a finger-bowl.

Early and late in the season when the *Arbacia* were not shedding readily, the ovaries were shaken over cheesecloth to obtain eggs. Late in the season, on a few occasions sperm were pipetted from ripe appearing testes which, however, were not shedding normally. Trial fertilizations came to be the rule; usually evidence from the fertilization membrane stage proved trustworthy. Relatively few experiments had to be discarded because of low percentage of cleavage. Eggs from one female only were used in any given comparison; usually eggs from one female sufficed for all experiments made in any given half-day. Late in the season eggs from a "good" female which had been opened about 9:30 A.M. were used for inseminations up to about 3:00 P.M. The fertilized eggs were allowed to stand 5 minutes and were then transferred to experimental conditions. All experiments were eliminated from consideration which did not show at least 95 per cent cleavage in the sparse populations and 90 per cent cleavage in the more densely populated drops. Usually the cleavage was well above 95 per cent in both.

When 90 or 110 per cent sea water was used in the experiment, the eggs were usually fertilized in this medium. At times, with these solutions, the eggs were fertilized in normal filtered sea water and centrifuged at the lowest speed shown by a power centrifuge for 15 seconds; this sufficed to throw them to the bottom. The supernatant sea water was poured off and the eggs were gently washed into the appropriate experimental medium.

After the first few days, initial handling of eggs and sperm was all done by Evans; two stop-watches were started by Allee at the moment of dropping the second drop of sperm suspension into the water containing eggs. All isolations and group transfers were made by Allee under a low power dissecting binocular. Typically, after standing five or six minutes, a part of the fertilized eggs were pipetted to a clean

Syracuse watch-glass recently rinsed with sea water. Using a hæmocytometer tube in which each of the ten units on the tube proper equaled 2 cu. mm., the approximate number of eggs needed were transferred to the experimental slides in drops of the selected size. Early in the work these transfers were made in the open air, sometimes, unfortunately, before open windows. Later all transfers were made to experimental slides temporarily housed in a moist chamber lined with wet filter paper. The separation was usually completed by 15 minutes after fertilization.

Cleaning

After the first few days, cleaning of glassware was done by Evans. The Syracuse watch-glasses into which the eggs and sperm were shed and the finger-bowls in which the eggs were washed and fertilized and all pipettes were washed in running cold tap water and boiled for at least 10 to 15 minutes. They were usually drained dry on paper towels. Occasionally when needed immediately they were dried with clean cotton towels. The hæmocytometer pipette used in isolations was similarly treated except that at times between isolations of different lots of eggs on the same half-day, it might be rinsed in fresh water and allowed to stand full of fresh water for as long as possible before the next transfer; frequently even under these conditions, this pipette was boiled. The eggs used in successive experiments were fertilized at least 15 minutes apart, hence any contamination could be easily recognized and discounted; very few occurred.

The glass slides were washed in warm soapy water to remove the vaseline and vacuum grease used in making seals. They were thoroughly rinsed in running tap water and were rinsed again in hot water and were usually boiled. They were polished with clean cotton towels.

Temperature

No attempt was made to control the temperature of the water in which the fertilizations took place. This water was usually the normal sea water freshly drawn from the laboratory tap, filtered and left standing in a large Florence flask. It was usually somewhat below air temperature at the time of fertilization. All observations were made in a room having north light only, hence there was no direct sunlight to interfere with room temperatures.

The small separate drops must have approximated air temperatures in a short time. These ran no higher than 25.5° at any time and were usually between 23–25°. When the air temperature was 20° or lower, the whole cleavage test was made at that temperature. In the hypertonic sea water experiments (110 per cent), room temperatures were also used. At other times the experimental slides were placed in cooling chambers for as much time as possible between the setting up of the experiment and the second cleavage. These chilling chambers varied from a rudely insulated makeshift refrigerator to an elaborately regulated chamber. They were usually held at 17–19° and normally were fairly constant for any given experiment. In one experiment the temperature went as low as 16°. An attempt was made to arrange the temperature so that first cleavage would come after some fifty minutes and second cleavage after about eighty-five minutes. Isolations were completed in about fifteen minutes and the slides were placed immediately in the cooling chamber, if that was the indicated procedure. Except in early experiments, dense and sparse populations were on the same slide and were accordingly subjected to the same temperatures throughout an experiment; hence more exact temperature regulation was not needed.

Test for Accuracy of Determination of Fifty Per cent Cleavage

In our first experiments we undertook to determine cleavage on living eggs. Accordingly we determined the relation between the time recorded by one of us for 50 per cent cleavage in living eggs, using our usual technique, as compared with the time similar eggs cleaved as determined by spaced killings of eggs kept under the same conditions. The mean error of determination of 50 per cent cleavage in eight cases was 10 ± 3 seconds.

Observations

The following experimental conditions were used and in this order:

Paraffined Slides.—Microscope slides with a shallow hollow ground cell were coated with paraffin. Two grades were used. The first had a relatively low melting point. The second was the highest grade, highest melting-point paraffin available in Woods Hole. There was no marked difference in the results obtained with the two grades. Melted paraffin was painted on the slides just before they were to be used and was removed, preparatory to washing them, after each experiment. One drop of water containing eggs was placed in the center of each depression and covered by a thin, somewhat curved watch-glass vaselined around the edge to make a small moist chamber. Typically 12 to 14 such slides made an experimental set. One had a drop containing approximately 100 to 200 eggs. Another had approximately half that number. The remainder held about two each. One person followed the cleavage of the two denser populations under a low power of a compound microscope while the other, seated nearby, followed it for the sparse populations. After the first experiment, the drops used contained 10 cu. mm.

These drops rounded into bead-like objects. Eggs near the periphery were sometimes difficult to see clearly. The slides which held the more densely populated drops were in the fingers of the observer more than was any one of the slides holding the sparser populations. This may have caused temperature differences between the two lots such as would favor the more rapid cleavage of the eggs in the former.

In the six experiments (see Table II) which meet the percentage of cleavage requirements, the time to 50 per cent first cleavage was 0.93 minutes and to second cleavage 2.17 minutes less in the denser populations of eggs than in the accompanying sparser populations. The statistical probabilities are 0.3 and 0.129 respectively. This means that neither of these differences are statistically significant.

Indications appeared that the paraffin retarded development. This was tested in two direct experiments in which approximately 21 hours after fertilization, the mortality was higher in the paraffined slides, particularly those with sparse populations. Accordingly this experimental method was discarded.

Clean Glass Slides.—The technique used resembled that just described except that no paraffin was used. With both of these sets of slides, it took about one minute to examine the lot of sparsely populated slides. Hence, to be significant, differences needed to be more than one minute provided all of the sparsely populated slides were to be inspected each time. Frequently as the critical time approached this was not the case since some of the slides would have all the few eggs present cleaved and could be discarded for the time being. In all, eight paired comparisons were made under these conditions. These showed a mean time to 50 per cent cleavage which averaged 1.53 minutes less time for the more crowded populations for the first cleavage and 2.2 minutes less for the second cleavage than for accompanying sparse populations (see Table II). These have statistical probabilities of 0.068 and 0.036 respectively. The latter is within the range of statistical significance as commonly interpreted.

Hanging Drops.—A central drop with from 11 to 1,000 eggs was surrounded by five similar drops holding in all from 6 to 28 eggs, usually about 10. As in the preceding series, the size of drops was kept uniform at 10 cu. mm. The drops were fenced from each other by vaseline lines to prevent their flowing together. Each slide, so arranged, was inverted over a salt cellar type watch-glass with ground glass upper surface which was vaselined to seal. A small amount of water in the bottom made an efficient moist chamber. This technique put sparse and dense drops under the eye of the same observer and on the stage of the same microscope. About one minute was consumed in making one complete inspection as cleavage approached 50 per cent. As elsewhere, if the 50 per cent cleavage point was reached in both dense and sparse populations during the same cycle of observations both were recorded as reaching this stage at the same time. The eggs in these hanging drops were located in the surface film exposed to unknown stresses. This constitutes the chief objection to the method.

Fifteen experiments which met standard conditions gave a mean time to 50 per cent first cleavage of 0.57 minutes faster in the more sparsely populated drops and to 50 per cent second cleavage of 0.24 minutes for the same eggs. These differences have a statistical probability of 0.215 and 0.84 respectively and accordingly the results have no statistical significance (see Table II).

Assembled Moist Chambers.—The assembled moist chambers consisted of a glass base 4 cm. sq. etched in the center with lines 1 mm. or less apart to form squares which aided in counting the denser populations of eggs. The vertical wall was formed by a glass ring ground on both surfaces. This ring was about 3 cm. in diameter and 4 mm. high. It was sealed to the base with vacuum grease and a plain glass cover was sealed on with vaseline. The base usually had vaselined lines to keep drops from running together. In much of the work, the vaselined lines were arranged in circles which limited the surface of the drops to about the same area. In one type of experiment, the denser population was placed in the center; the five surrounding drops held normally two eggs each; in three cases there were as many as 15 to 23 eggs in one of these sparsely populated drops. The time consumed in making a complete cycle of observations was about that with the preceding methods. Later only two drops were used, one with a dense population which might run up to an estimated 8,000 in 20 cu. mm. Nearby, perhaps connected with the denser lot of eggs, perhaps isolated, would be a similar drop with a sparse population. The time from the determination of 50 per cent cleavage in the denser population until a count of cleavage could be made in the sparser one was usually 15 seconds or less. All the later experiments were made using this last method.

The seals on such assembled moist chambers had to be carefully made to avoid evaporation. These seals allow for ease of cleaning and the possibility of rapidly changing clouded covers. Evaporation from the experimental drops was frequently lessened by the addition of unoccupied drops of sea water in the free spaces. Even so, with the small drops and the change of covers made necessary by their clouding on removal from cooling chambers (see below), some evaporation must have taken place. It is difficult to determine whether the evaporation is equally distributed through these small drops. The drop isolated first, other conditions being equal, should show the greatest amount of evaporation. There is evidence that hypertonicity is a potent factor in retarding the time of cleavage of echinoderm eggs and since in many of these experiments the eggs in the more densely populated drop tended to cleave first, these were isolated first. Here and elsewhere whenever it was impossible to set up conditions exactly equivalent for dense and sparse populations, the sparser population was favored. This necessitated the isolation of the dense group last when hypotonic sea water was used, since under such conditions the longer the drops stood exposed to air, the more nearly they approached normal concentration.

Table I

A summary of time to 50 per cent cleavage with two connected or closely associated drops, one containing a very dense and the other a sparse population. All were tested in normal sea water.

Crowded Drops				Sparsely Populated Drops						
No. Eggs	Minutes to Fifty Per Cent Cleavage		Percentage Cleaved	No. Eggs	No. Followed	Minutes to Fifty Per Cent Cleavage		Percentage Cleaved	Cu. Mm. in Drop	Temperature ° C.
	I	II				I	II			
3500	63.25	94.25	98+	17	17	64.83	97.50	100	10	18–25
4000	61.00	94.50	99+	130	28	62.25	96.67	99+	10	16–24
1600	67.83	100.04	99+	250	24	70.67	104.41	99+	10	16–24
2100	68.33	101.25	99+	11	11	70.75	103.41	100	10	16–24
2000	71.83	105.00	99+	41	21–30	74.00	108.08	100	10	16–24
1800	55.41	84.00	100	40	10	56.17	86.58	100	20	21–21.5
4000	52.17	80.50	100	800	10	60.25	90.25	100	20	21–21.5
5000	62.91	95.75	99	600	16	64.25	97.75	100	40	19
2500	58.67	—	99	800	16	61.00	—	100	40	19
6000	62.33	96.50	96	15	12	63.75	99.08	100	20	19–20
8000	61.25	95.00	96	17	12	63.25	97.25	100	20	19–20
6500	61.58	95.25	95	14	12	61.83	96.25	100	20	19–20
2800	61.33	94.58	95	23	12	61.17	94.83	100	20	19–20
3831	62.145	94.72	98	212	16	64.174	97.67	100−	19	

Similar experiments were performed with normal sea water, with 110 per cent and with 90 per cent sea water. With the 110 per cent sea water no cooling was used to slow down development.

The results obtained with the two drop technique are given in some detail in Table I and all the work done with the assembled moist chamber is summarized in the last four lines of Table II. In all these cases the time to 50 per cent second cleavage was significantly less for the more crowded eggs. The decreased time ranged from 2.17 to 3.16 minutes and the statistical significance from 0.0134 to 0.0002. As usual, there was less difference in the time elapsed before 50 per cent first cleavage. With 90 per cent sea water the results were slightly negative (0.03 minutes, probability 0.67). In two other sets of experiments this time difference increased to 1.9 and 2.03 minutes earlier cleavage for the more crowded drops with statistical probabilities of 0.0084 and 0.0036 respectively. The fourth comparison made (line 4, Table II) gave positive results which, however, lack statistical significance for first cleavage although there is good significance for the second cleavage.

TABLE II

A summary of differences in time to 50 per cent first (I) and second (II) cleavage with dense and sparse populations of Arbacia eggs.

No. of Experiments	Method	Mean Difference in Minutes		Statistical Probability	
		I	II	I	II
6	Paraffin	0.93	2.17	0.3*	0.129*
8	Glass slides	1.53	2.2	0.068*	0.036
15	Hanging drops	−0.57	−0.24	0.215*	0.84*
21-23	Moist chamber	0.54	2.7	0.194*	0.002
17-18	Do. 110 per cent	1.9	3.16	0.0084	0.0002
10	Do. 90 per cent	−0.03	2.17	0.67*	0.0134
12-13	Do. connected drops	2.03	2.95	0.0036	0.0014
True means and combined significance		0.88	2.23	0.053	0.0016

* Results taken alone are not statistically significant.

The use of two connected drops, one with a dense and the other with a sparse population of eggs, sometimes permitted the observers to determine the time to 50 per cent cleavage in different regions with different densities. Two such determinations (in which Allee was observing and Evans was recording) are summarized in Fig. 1.

Averages of the time to 50 per cent first or second cleavage for the experiments summarized in Table II have lessened value because of the varieties of techniques used. The mean differences are based on individual paired experiments in any one of which the difference in density of the egg population was the only factor known to vary. If the true mean, considering the number of individual tests, is taken, there is a mean difference in time to 50 per cent first cleavage of 0.88 minutes with the eggs in the denser populations cleaving first. This has a statistical probability of 0.053 which is slightly above the conventional limit of statistical significance. By second cleavage, this difference has increased to a mean of 2.23 minutes and the statistical probability has increased to 0.0016.

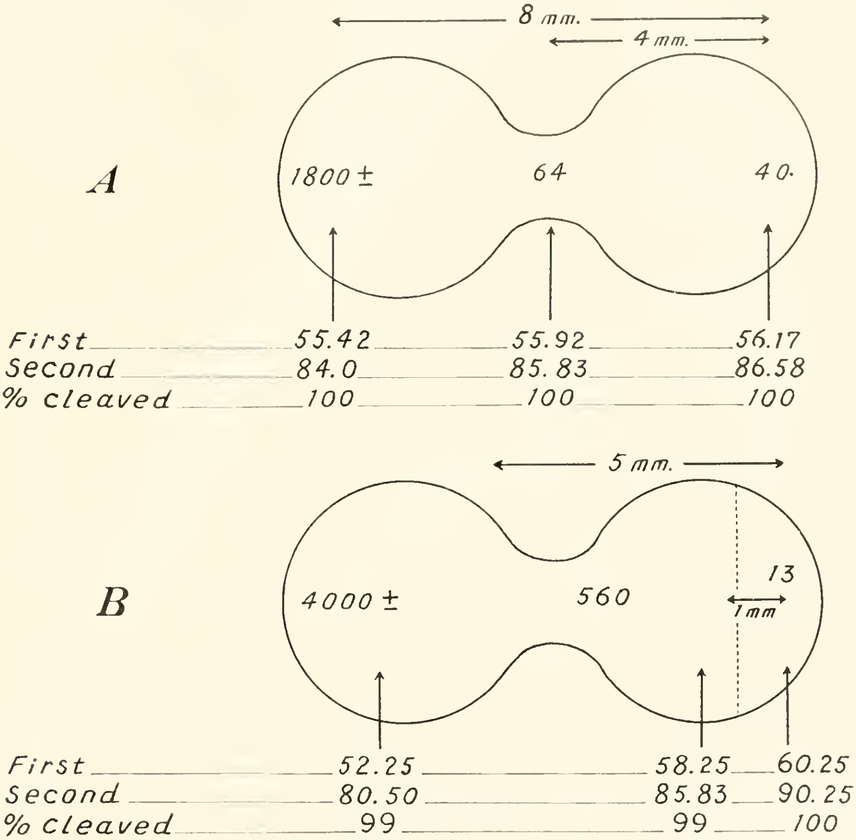
8 mm.
4 mm.
A
1800 ±
64
40·
First 55.42 55.92 56.17
Second 84.0 85.83 86.58
% cleaved 100 100 100
5 mm.
B
4000 ±
560
13
1 mm
First 52.25 58.25 60.25
Second 80.50 85.83 90.25
% cleaved 99 99 100

FIG. 1. Diagrams of the connected drops used in two different experiments. Each drop contained 20 cu. mm. The measurements give distances between points indicated by the double arrows. Figures below the diagrams, unless otherwise indicated, give time in minutes to the designated cleavage.

The values just given include those with hanging drops where the relations are obviously different from those obtaining in the other types of experimentation. If the hanging-drop experiments are omitted, these true means become 1.17 and 2.68 minutes with "p" values of 0.016 and 0.0000 respectively. Since these hanging-drop experiments do not support the evidence from the rest of the work to date and since we do not know why this is the case, it is probably better to include these in the summaries. At least this is the conservative procedure and is in line with the general practise in these experiments of weighting the results against the experimental trend whenever it was impossible to be absolutely impartial.

Thus far in this account we have been dealing mainly with time relations with regard to 50 per cent cleavage. It is profitable to examine the assembled data from another approach which may be indicated by the following question: How many cases were there in which there was evidence of a shorter time elapsing between fertilization and 50 per cent cleavage in the denser population of eggs and in how many cases did the opposite hold true? The answers are given in Table III properly subdivided among the different possibilities. In the preparation of this table, unless there was a clear difference between the time to 50 per cent cleavage such that the difference could not be the result of error in observation, the case was recorded as showing no difference. Table III shows correctly that our data clearly indicate speedier cleavage in the more densely populated drops.

TABLE III

Relations between first and second cleavages.

Evidence of Stimulation in Denser Population (D)	Without Hanging Drops	With Hanging Drops
1. D cleaved first in both cleavages	46	48
2. First cleavage a tie; D faster in second	24	27
3. D slower in I; faster in II	5	5
4. Sparse faster in I; second even	5	5
Totals	80	85
Evidence of Retardation in Denser Population		
5. S cleaved first in both cleavages	5	8
6. First cleavage a tie; S faster in II	0	5
7. D faster in I; S faster in II	0	0
8. S slower in I; second, even	1	2
	6	15
9. No observed difference	13	17
Totals	19	32

These experiments taken together demonstrate fairly conclusively that there is a difference in time to 50 per cent of first and second cleavage for *Arbacia* eggs in relatively sparse and in relatively dense population. The data collected are clearly statistically significant on this point for the second cleavage, less so for the first. It is necessary to inquire into the degree of crowding which will bring about this result. Observations on this point were made from time to time during the progress of the experiments. Those obtained later in 1934, using the paired drop technique with one drop densely crowded with eggs closely associated or even actually directly connected by a narrow isthmus with a similar drop which contained but few eggs, are summarized in Table IV.

TABLE IV

Numbers present with relation to the crowding effect.

No. Experiments	Dense No. eggs	Sparse No. eggs	Difference in Fifty Per Cent Cleavage		Probability		Size Drops Cu. Mm.	Eggs Followed Sparse
			I	II	I	II		
12–13	1600+	800−	2.04	2.95	0.0036	0.0014	10–40	10–30
15	65–164	5–24	0.23	1.26	0.7	0.045	20	5–24
14	22–56	5–18	0.08	−0.3	0.38	0.426	20	5–18

In this table the difference in time to 50 per cent first and second cleavages are compared for crowded drops containing over 1,600 eggs as compared with that in more sparsely populated drops containing 800 eggs or less. In reality the listing of these hundreds of eggs in the sparser populations is hardly fair for in all cases the eggs watched in such drops were out of contact with each other while those in the densely populated drops were closely crowded. Under these conditions (which have already been discussed in some detail) the eggs in the more crowded drops cleaved significantly earlier than did those in the accompanying sparsely populated drops of equal size. When the more crowded drop contained but 65–164 in a 20 cu. mm. drop and the accompanying drop held 5–24, the difference in time to first cleavage was insignificant but the denser eggs cleaved slightly sooner at second cleavage and the difference of 1.26 minutes is just within the upper limit for statistical significance. When the population in the more dense drop is still further reduced and that in the sparsely populated drop remains about the same, the difference disappears for both cleavages.

Factors Known to Retard Cleavage

The factors known to retard cleavage which may have operated in one or more of these experiments are:

1. Hypertonic sea water.
2. Lowered temperature.

3. Contamination with cœlomic fluid.
4. Contamination with fragmented eggs.
5. Increased amount of metabolic wastes in the water.

Of these, there was no opportunity for differential contamination with cœlomic fluid in the different parts of a given test. The precautions taken against hypertonicity have already been outlined and while they were not always successful when differential treatment was unavoidable as in the time of setting up of the various drops, especial care was taken to load the experiment against the trend of experimental findings.

As regards temperature differences, when one observer examined the one or two slides holding dense populations and the other handled the half dozen or more with sparser ones, there was the possibility of the former having a slightly higher temperature from the more steady contact with the fingers of the observer. This possibility was eliminated in the tests with the assembled moist chambers which constitute the majority of the experiments reported here. Furthermore, these latter experiments yielded greater and more consistent differences than were found under conditions that might have been suspect, hence there is no evidence that the observed differences are the result of an externally induced temperature differential.

There is, however, a possibility that the high rate of oxidation of the larger number of newly fertilized eggs confined in a small space may produce a temperature differential sufficient to account for the observed results. No tests of this possibility have been made to date. The best evidence that can be cited in its favor is that a slight differential increase in temperature would produce the difference which we have found.

The isolated eggs were definitely favored as regards the presence of fragmented or immature eggs. Allee found it psychologically difficult to select other than good eggs for the relative isolation of the sparse lots while in scooping up from 50 to 8,000 eggs for a more densely populated drop, no such selection was possible. Hence the sparsely populated drops contained less debris both absolutely and proportionally than did those with the large number of eggs.

Overcrowding.—In the most densely populated drops used in the regular experiments, eggs were present in about the proportion of 0.5 cc. of centrifuged eggs (lowest speed with power centrifuge for one minute) to six cc. of water. In Syracuse watch-glasses with this concentration there was reduced cleavage. In drops of 10 or 20 cu. mm., the eggs are nearer to the surface of the drops and while they may be piled three or four deep in the center, cleavage goes almost as well as in the finger-bowl controls. When the same concentration is placed in a watch-glass or finger-bowl, i.e. in the same concentration when the eggs are all stirred up, the eggs settle to the bottom in a much more dense mass than any we worked with on the slides and under these conditions they were definitely over-crowded.

Mass Protection.—In an attempt to avoid the added evaporation incident to changing clouded covers, a microscope stage-cooling device was developed. This consisted of a brass quadrangle about the length of the microscope stage and somewhat narrower and about three centimeters high. Glass tops and bottoms were sealed on with DeKotinsky cement. The upper glass plate had patches of etched lines to facilitate counting and accommodated two of the 3 cm. glass rings which were sealed to the glass with vaseline. Intake and drain tubes were soldered to the brass quadrangle and were attached to the sea water supply since this was the coolest water available. This was further cooled by running it through a copper coil placed in a bucket of ice water. The drops were placed on the glass as usual except that during isolation, a covering watch-glass was used for a partial moist chamber. After covering the drops with the usual vaseline-sealed, glass cover, the water was turned through the chamber and the temperature fell. With the room temperature at 24°, in one instance, water emerging from the cooling device showed 12°; it was usually held at from 17–19°. This device was discarded when it appeared that there was difficulty in proper cleaning of the surface to which the eggs were exposed and no data secured by this means have been included in the preceding tables.

Some twelve experiments were tried using this gadget. The mean difference to 50 per cent cleavage for those that cleaved was 5.0 minutes for first and 5.62 minutes for second cleavage, with " p " values of 0.024 and 0.0052 respectively. Four accompanying experiments made in the assembled moist chambers already described had differences to 50 per cent first and second cleavages of 0.31 and 2.8 minutes respectively with the latter difference statistically significant. In two cases with the stage-cooling device, sparse populations of 6 and of 10 eggs failed to cleave and in two other experiments sparse lots of 24 and of 34 did not reach 15 per cent cleavage. The accompanying densely populated drops developed to or beyond the blastula stage and in three of the four comparisons just made, many in the more crowded lots were actively swimming after 24 hours. These data strongly suggest the presence of some toxic contaminating substance which was not completely removed by the methods used in washing these stage-cooling devices. Further tests showed that in connected drops gradients of resistance could be demonstrated which depended on the numbers present, the more eggs within the limits tested, the greater the percentage of cleavage and the further development would proceed before death. Such mass relations appear to be closely related to the mass protection from toxic materials which has been repeatedly demonstrated (*cf.* Allee, 1931, 1934).

Could this have been the explanation of the more rapid cleavage observed in the denser populations? There is a suggestion that it may have been a factor in the paraffined slides, which, however, is not borne out by comparative studies unless there was some other toxic agent acting similarly in the remainder of the experiments. To suppose that there was mass protection from toxic materials in the other experiments would imply either some sort of toxic emanations from the glass itself or from some chemical previously in contact with it (Richards, 1936), or that traces of the vaseline and/or vacuum grease remained over from the washing and were poisonous, or that some of the soap from the mild suds used remained after the extensive and careful rinsing. There is no evidence for the presence of toxic materials from any of these sources all of which were considered as possible means of experimental error before this set of experiments was begun. Direct tests made by using water which had stood over masses of broken glass showed no difference in development as compared with similar cultures in ordinary sea water. Direct tests for toxic effects from vaseline and from the vacuum grease used made by coating slides with these substances and placing drops with different numbers of eggs on them, yielded no evidence of contamination from this source. Hence we concluded (and later experiments to be reported in another paper justify the conclusion) that the differential results obtained are not produced by mass protection such as was demonstrated to be operating in the experiments made with the stage-cooling device.

There is also internal evidence from the experimental results reported in Table II that something more is happening in these experiments than would be expected from the simplest of mass relations whether mass protection or otherwise. The data given there show that the mean time to 50 per cent first cleavage for all the experiments was 0.88 minutes (omitting hanging-drop experiments, 1.17 minutes) and to second cleavage was 2.23 minutes (omitting hanging drops, 2.68 minutes). The mean time to 50 per cent first cleavage was 57 minutes and to the same stage in second cleavage was 85 minutes.

If this were a case of simple mass relationships one would expect the acceleration of 0.88 minutes during a period of 57 minutes to continue at the same rate during the following 28 minutes to second cleavage. At this rate the total acceleration would approximate 1.31 minutes, which is not the case. Even when one corrects for the fact that for the first ten minutes after fertilization, on the average, the eggs which are to make up the isolated or sparse populations are in fairly dense lots, the expected acceleration on the basis of uniform, simple mass action would be 1.40 which even yet is far from the observed value. Even omitting the hanging-drop experiments, which we decided above not to do, and using the basis of calculation which will give highest results, simple mass action would call for an acceleration by second cleavage of 1.87 against the observed value of 2.68 minutes. These relationships indicate that the observed phenomena are not based directly on the simplest sort of mass physiology.

Other possible causal factors which deserve investigation include the effect, if any, of increased carbon dioxide, of supernatant water from eggs both before and after cleavage, and of mitogenetic rays.

SUMMARY

1. Other conditions being equal and under a variety of experimental conditions, eggs of *Arbacia punctulata* cleave more rapidly when in relatively dense as opposed to relatively sparse populations. The decreased time to first cleavage in the dense populations was 0.88 minutes and to second cleavage was 2.23 minutes. The first difference is probably not statistically significant; the second value is clearly significant.

2. Among other conditions, these relations were observed when some thousands of eggs in a drop of 20 cu. mm. of sea water were connected by a narrow strait with a similar drop holding some few tens or even a few hundreds of eggs.

3. If the eggs were crowded together too densely, the time to first and second divisions was definitely retarded and the percentage of final cleavage was reduced.

4. When 22 to 56 eggs were placed in one drop of 20 cu. mm. connected by a strait with another containing 5 to 18 eggs, no difference in cleavage rate was observed.

5. The observed differences are not a result of differential temperatures externally imposed, differential hypertonicity or hypotonicity, contaminations with cœlomic fluid or with fragmented eggs.

6. Such results may be obtained by mass protection from toxic materials. There is, however, no indication that differences here reported were so caused.

7. Although no supporting evidence is presented here, the results may conceivably have been the result of differential temperatures produced by the high rate of oxidation of the massed eggs in a small space,

by chemical emanations from the eggs, including carbon dioxide, by stimulation from mutual contact or by mitogenetic rays. Discussion of these problems is reserved for the present.

8. In addition to their intrinsic interest, the results provide another instance of physiological activities which proceed more rapidly at an intermediate optimum than when either too few or too many are present.

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FEEDING RATE OF CALANUS FINMARCHICUS IN RELATION TO ENVIRONMENTAL CONDITIONS[1]

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OBJECTIVES

The amount of food available to a plankton-feeding animal is determined by the concentration of suitable food material in the water, and by the rate of the animal's feeding activity. The calanoid copepods, like many other zoöplanktonts, are generally considered to feed by filtering out particles from a current of water generated by the animals (Cannon, 1928). By determining the number of food organisms removed by an animal from a suspension of known concentration, the volume of water which has been filtered clear of the organisms can be calculated and, if the chemical composition of the organisms be known, the amount of nutriment made available to the animal may be estimated. Preliminary measurements of the filtering rate of *Calanus finmarchicus* have been reported by Fuller and Clarke (1936). Further investigation of changes in the feeding rate induced by varying environmental factors of ecological importance was expected to yield information useful in the quantitative study of aquatic food cycles. Diatom concentration, light and temperature were expected to be variable factors in the sea and were chosen for study.

METHODS

In the experiments here reported the rate of feeding of the copepod *Calanus finmarchicus*, collected in Vineyard Sound near the whistle buoy, was measured in a suspension of the diatom *Nitzschia closterium* (Plymouth strain). It seems probable that *Nitzschia* is a suitable food for *Calanus*, as Crawshay (1913–15) kept individual copepods alive for as long as 80 days in persistent cultures of this diatom. Allen and Nelson (1910, p. 470) reared *Calanus* from eggs to copepodid stages in a mixed culture in which *Nitzschia* was predominant.

Three stage V *Calanus* were placed in 15 cc. of sea water containing a known concentration of diatoms. Changes in concentration were followed for two to four days, counts being made in a hemacytometer.

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[1] Contribution No. 132 of the Woods Hole Oceanographic Institution.

A series of counts on successive samples showed fair agreement with the theoretical Poisson distribution. The small excess variation found is attributed to the difficulty of shaking the diatoms to obtain even distribution without injuring the copepods. Enough diatoms were counted for each sample to give a result statistically valid to between 5 and 10 per cent. Under the experimental conditions this is the maximum precision obtainable. In these experiments a counting error of 10 per cent causes a discrepancy of about 30 per cent in the estimation of the filtering rate. Obviously only large variations can be satisfactorily studied.

TABLE I

Feeding rate at different diatom concentrations

Expt. No.	Date begun	Cells per cc.		Duration	W_{24}	ΔN_{24}
		C_1	C_2			
				hours	*cc.*	
47	Aug. 18	1,875	1,140	42	1.32	2,100
48	Aug. 18	1,875	1,155	42	1.28	2,060
63	Aug. 23	19,000	15,400	38	.66	11,400
64	Aug. 23	19,000	13,100	38	1.17	16,100
14	July 9	64,700	27,000	81	1.30	55,000
15	July 9	64,700	27,700	81	1.25	55,000
71	Aug. 26	190,000	147,000	38	.81	136,000
72	Aug. 26	200,000	133,000	38	1.29	212,000
11	July 3	335,000	86,000	84	1.61	356,000
10	July 3	375,000	220,000	84	.43	221,000
8	June 29	390,000	130,000	84	1.32	371,000
7	June 29	410,000	230,000	84	.60	256,000
				Average	1.09	

Calculation of the amount of water, W_x, swept free of diatoms by each copepod in x hours was made by means of an equation derived as follows:

$$dN = -dWC \tag{1}$$

$$dC = dN/V \tag{2}$$

$$dW = VdC/C \tag{3}$$

$$W_x = V \ln C_1/C_2. \tag{4}$$

N represents the number of diatoms per copepod; V the volume of water per copepod; C_1 and C_2 respectively the concentrations of diatoms at the beginning and end of the period of x hours.

The number of diatoms eaten in x hours per copepod, ΔN_x, is given by the equation:

$$\Delta N_x = V(C_1 - C_2). \tag{5}$$

Parallel control suspensions were always counted to determine whether the diatom numbers were varying independently. If a control changed greatly during the experimental period, the results of the corresponding experimental series were discarded. If the change was small, the final concentration of diatoms in the control was substituted for C_1 in equations (4) and (5). This substitution assumes that the diatoms in the experimental containers increased or decreased independently exactly as those in the control. This is, of course, only an approximation.

Observations were also made on the production of fecal pellets. Since these pellets vary greatly in size no quantitative relationship could be obtained between the number of diatoms ingested and the number of pellets ejected. However, the formation of these excreta was a useful check on the reduction of diatom numbers observed directly.

EFFECT OF CHANGING DIATOM CONCENTRATION

Table I gives the results of experiments with different concentrations of diatoms. In the lowest concentration the diatoms were concentrated by the Nielsen-von Brand (1934) method before counting. These experiments were carried out at 13° C., and the animals were shielded from direct sunlight. The values for W_{24} and N_{24} in the table represent means over a period of several days. Diatom concentration often remains stationary for a day or more, thus indicating that feeding is not a continuous process. The maximum rate of filtration is higher than these values. The highest rate observed was in Experiment 11 where over a period of 14 hours W equalled 2.87 cc. which would give a W_{24} value of 4.9 cc. There appears to be no correlation between the concentration of food and the filtering rate. A comparison of the filtering rates of animals collected at different times during the summer yields no greater differences than a comparison of animals collected at the same time and used in parallel experiments (e.g. Nos. 10 and 11). The indication is that *Calanus*, though it sometimes does not feed actively, under otherwise constant conditions filters a definite volume of water per day, and obtains nutriment in direct proportion to the concentration of food particles.

EFFECT OF LIGHT ON FEEDING RATE

In certain experiments there appeared to be a diurnal feeding rhythm, removal of diatoms taking place most rapidly at night. This is in accord with the observation of Marshall (1924), who notes that *Calanus* captured by tow-net hauls in the early morning had full guts, while those caught later in the day often had no food in their alimentary tracts. Figure 1 represents the course of two experiments (8 and 11) which show this rhythm. It is not, however, an invariable effect, as is shown by the course of Experiment 15, also shown in Fig. 1. Marshall likewise found that during the summer feeding occurred during the day as well as at night.

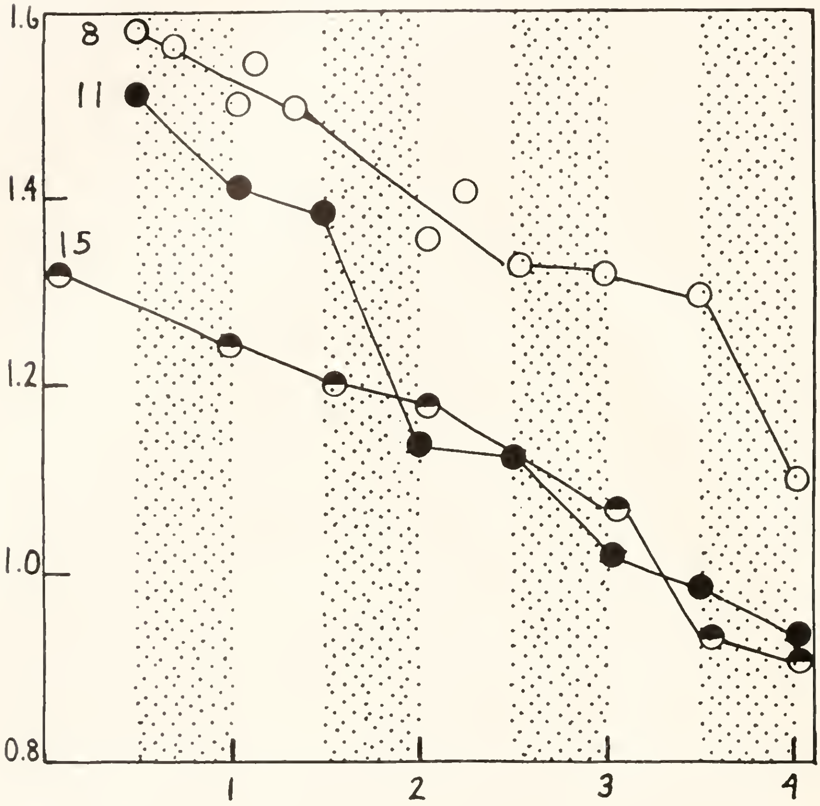

FIG. 1. Diurnal feeding rhythm of *Calanus*. Stippled areas represent hours between 8 P.M. and 8 A.M. The scale of Experiment 15 is adjusted by adding 0.5 to logarithms of cell numbers. Ordinates: *Logarithm-cells per 0.1 cu mm.* Abcissæ: *Time in days.*

Experiments 26 and 32 differed from those reported above in being kept in darkness. Table II summarizes the results of these two experiments.

TABLE II

Feeding rate in dark

Expt. No.	Date begun	Cells per cc.		Duration	W_{24}	N_{24}
		C_1	C_2			
				hours	*cc.*	
26	July 16	54,000	32,000	90 48*	.27 .76*	45,000
32	July 30	61,000	20,000	65	1.8	76,000

* Animals actually fed only during last 48 hours of experiment. W_{24} value calculated for this period.

The results do not differ significantly from the average given in Table I, although there are admittedly too few cases to permit a definite answer. An attempt was made to study the effect of continuous light. The animals did not feed during the two days they were observed, but they were old stock in poor condition, and no reliance should be placed on this observation. Unfortunately it was impossible to collect more *Calanus* to make satisfactory experiments.

EFFECT OF TEMPERATURE ON FEEDING RATE

Experiments were carried out at 8° C. and at approximately 3° C. The latter temperature was not precisely controlled as the containers were placed in a refrigerator in general laboratory use. Both series were kept in the dark.

Feeding is much slower at 3° but still goes on with the formation of small compact fecal pellets. The very low filtering rates observed in Experiments 75 to 79 may be explained by the fact that the animals used were collected late in the season when the *Calanus* population was receding rapidly, and had perhaps stopped eating. The filtering rate in Experiment 80, in which the same stock was used, is seen to be much slower than that in Experiments 55 and 56. The high value for W_{24} in the latter experiments seems to indicate that 8° C. is somewhat more favorable for feeding than 13°. Excluding Experiments 75 to 80, the average values of W_{24} at 3°, 8° and 13° are respectively, 0.35, 2.83 and 1.09 cc.

ORGANIC NITROGEN CONTENT OF NITZSCHIA AND OF PLANKTON

Through the kindness of Dr. Theodor von Brand, an analysis of the organic nitrogen content of *Nitzschia* was made by the method he has developed for small amounts of plankton (von Brand, 1935). *Nitzschia* contains 0.9 microgram of nitrogen per million cells. Assuming the carbon-nitrogen ratio in this diatom to be equal to that generally found in marine phytoplankton, approximately 8 : 1, we have 7.2 micrograms of carbon per million cells. Analysis of the fecal pellets of *Calanus* fed on *Nitzschia* showed that 0.55 microgram of nitrogen appeared in the feces for each million cells ingested. Roughly, about half the organic nitrogen was retained by the copepods.

Marshall, Nicholls and Orr (1935) have calculated from measurements of oxygen consumption the daily nutritive requirement of a stage V *Calanus* in summer as equal to 13 micrograms of carbohydrate. Assuming all the nitrogen of *Nitzschia* to be in protein, and the excess carbon to be in fat, one may calculate the number of cells which would supply an equal amount of energy. If the protein is assumed to contain 16 per cent nitrogen and 50 per cent carbon; the fat to contain 75 per cent carbon, we have:

TABLE III

Effect of temperature on feeding rate

Expt. No.	Temp.	Date begun	Cells per cc.		Duration	W_{24}	N_{24}
			C_1	C_2			
	° C.				*hours*	*cc.*	
42	3°	Aug. 18	263,000	215,000	90	.38	64,000
43	3°	Aug. 18	263,000	223,000	90	.32	53,000
55	8°	Aug. 22	85,500	30,000	42	2.88	159,000
56	8°	Aug. 22	85,500	31,000	42	2.78	156,000
80	8°	Aug. 31	24,000	14,000	65	.98	18,500
(75, 76) (79)	8°	Aug. 31			85	.00	0

$0.9 \times 0.16 = 5.62\ \gamma$ protein containing $2.8\ \gamma\ C$.
$7.2 - 2.8 = 4.4\ \gamma\ C$ in fat
$4.4 \times 0.75 = 5.87\ \gamma$ fat.

Proteins and carbohydrates have equal energy values per unit of weight. Fat has 2.25 as great an energy content as carbohydrate. Calculating fat and protein in terms of carbohydrate, we obtain:

$5.87 \times 2.25 = 13.2$
5.6
$18.8\ \gamma$·weight of carbohydrate equivalent in energy content to a million *Nitzschia* cells.

$\frac{13}{18.8} \times 1{,}000{,}000 = 690{,}000$ number of *Nitzschia* cells which would contain theoretical food requirement.

The maximum amount of water filtered per day (W_{24}) in any experiment was 2.88 cc. A concentration of 240,000 cells per cc. would be required if *Calanus* is to obtain its theoretical food requirement from this volume of water, even if all the organic matter is utilized. Probably this value should be doubled since only half the nitrogenous organic matter appears to be assimilated. In any case this is a much higher concentration of phytoplankton than ever occurs in nature, but since the size of phytoplankton cells varies so greatly, comparisons based on cell numbers are worthless. It is, however, possible to determine the organic nitrogen content of the particulate matter in sea water, and to compare this with the food requirements of *Calanus*. Table IV summarizes the results of two sets of analyses by Dr. von Brand of the particulate matter suspended in Vineyard Sound water. All macroscopically visible organisms were removed from the August 14 samples before analysis. This was not done for the July 2 samples.

TABLE IV

Organic N in particulate matter in Vineyard Sound water

Date	Depth	Temperature	N
	meters	° *C.*	*per liter micrograms*
July 2	0	17.8	15
	15	13.5	27
	30	9.1	13
August 14	0	20.0	38
	15	18.0	42
	30	12.0	19

The maximum value found was 42γN per liter, a nitrogen concentration equal to that of a *Nitzschia* culture containing 46,700 cells per cc. This is so far above the average number of diatoms per cc. in Vineyard Sound—the figure is probably close to 100—that, even allowing for the fact that one large cell is the equivalent of many *Nitzschia*, it appears probable that the major portion of the particulate matter at this station was not in the form of diatoms. The remainder, detritus or other organisms, is presumably of great significance as a source of food.

Even in this region of high nitrogen content 2.88 cc. of Vineyard Sound water contained less than one-fifth the theoretical food requirement, and in all probability not over one-tenth this amount since half the nitrogenous matter is believed inassimilable, and some of the particles were probably too large or too small to be captured. Yet *Calanus* survives and grows in this region. Two explanations are possible: (1) *Calanus* in nature requires less food than that calculated from laboratory experiments on oxygen consumption, or (2), *Calanus* in nature filters a greater volume of water and thus obtains more than the estimated amount of food.

NUTRITIVE REQUIREMENTS OF CALANUS

Knowing the rate at which *Calanus* feeds on *Nitzschia*, it should be possible to supply the animals with known concentrations of diatoms, observe the minimum concentration at which growth takes place, and calculate the food requirement in terms of planktonic organic nitrogen. Experiments were set up in which 15 to 20 *Calanus* were placed in 250 cc. of Berkefeld filtered sea water containing definite concentrations of *Nitzschia*. The concentrations chosen together with the amount of nitrogen per cc. in each case were:

TABLE V

Survival and moults of Calanus fed on Nitzschia

Expt. No.	Nitzschia cells/cc.	Initial population		Successful moults	Deaths in moult	Deaths not in moult	Percentage reaching moult	Average survival
		No.	Stage					*days*
B1	300,000	2 12 5	III IV V	0 1 0	2 8 1	0 3 5	63.2	8.9
B2	150,000	11 4	IV V	1 0	3 1	7 3	33.3	9.7*
B3	30,000	14 4	IV V	0 0	5 2	9 2	38.9	9.5
B4	15,000	8 11	IV V	0 0	3 5	5 5	41.1	10.4*
B5	0†	10 6	IV V	0 0	1 2	9 3	18.7	11.0*
B6	Harbor water	4 16	IV V	0 0	3 9	1 7	60.0	7.2

* In each of these experiments one stage V still living after 19 days.

† All attempts to moult near end of experiment. Examination showed minute protozoa present. None in Experiments B1–B4.

(B1) 300,000 cells and 0.27γN per cc. representing a concentrated food supply with the theoretical amount of nutriment contained in 2.5 cc.

(B2) 150,000 cells and $0.135\gamma N$ per cc., half the above.

(B3) 30,000 cells and $0.027\gamma N$ per cc. representing an approximation to the concentration of organic nitrogen found in Vineyard Sound.

(B4) 15,000 cells and $0.014\gamma N$ per cc., half the above.

(B5) Berkefeld filtered water—no diatoms.

(B6) Water collected from Woods Hole Harbor.

The medium was changed daily except Sunday, and dead animals were removed at each observation period. Animals were transferred by means of a lifter of bolting cloth or in a wide-mouthed pipette. In order to study growth, stage IV *Calanus* were used insofar as they were available. Survival was uniformly poor compared with experiments which were reported previously by Fuller and Clarke.[2]

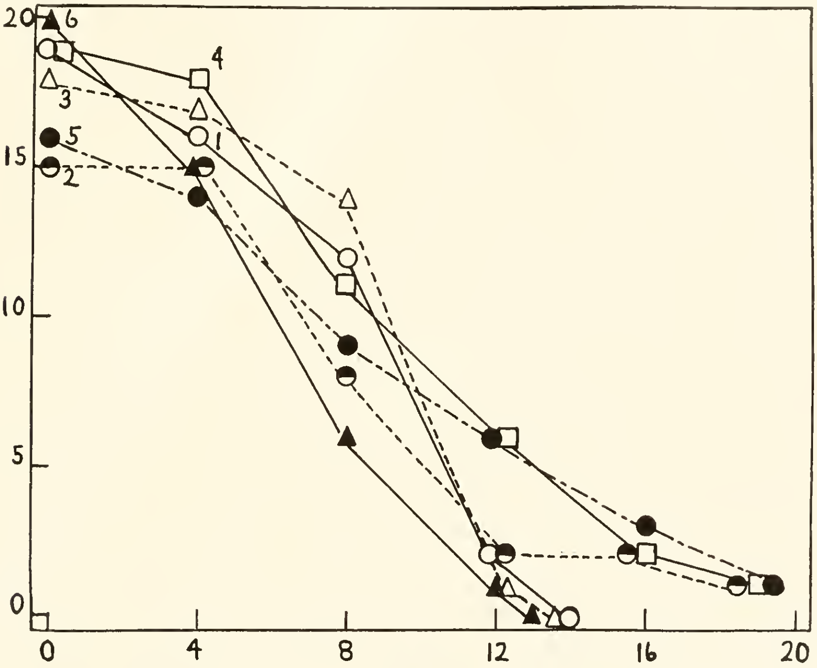

FIG. 2. Series B. Survival of *Calanus* fed on different concentrations of *Nitzschia*. B1—300,000 cells/cc.; B2—150,000 cells/cc.; B3—30,000 cells/cc.; B4—15,000 cells/cc.; B5—Berkefeld filtered water; B6—water from Woods Hole Harbor. Ordinates: *Number of survivors.* Abcissæ: *Time in days.*

These experiments are summarized in Table V and Figs. 2 and 3.

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[2] Clarke and Zinn found that in both 1935 and 1936 there was a sharp reduction in the *Calanus* population in Vineyard Sound during the latter part of August. The animals were either being carried away, or were dying off. This may explain the high mortality in these experiments.

The previous observation that ecdysis is a critical period for *Calanus* is again brought out by the data for "deaths in moult." It is believed, however, that the attainment of the moulting stage represents true growth, and indicates that the animals are feeding, since ecdysis does not occur in starved animals (Fuller and Clarke, p. 312). If the attainment of the moulting stage—whether successful or not—be taken as the criterion, growth is most rapid in the most concentrated diatom suspension and in harbor water in which media 63.2 and 60 per cent, respectively, of the original population reached the moulting stage. Growth rates in the other diatom suspensions are essentially equal (33–41 per cent), while the few moults which occurred in Berkefeld filtered water (19 per cent) are probably due to a protozoan infection of the culture which developed during the last few days of the experimental period and furnished a food supply for the copepods. There is a definite negative correlation between the average survival period in each experiment and the percentage of animals attaining the moulting stage. This is attributed to the fact that when growth is slower, the critical period of moulting is reached later and average survival increases.

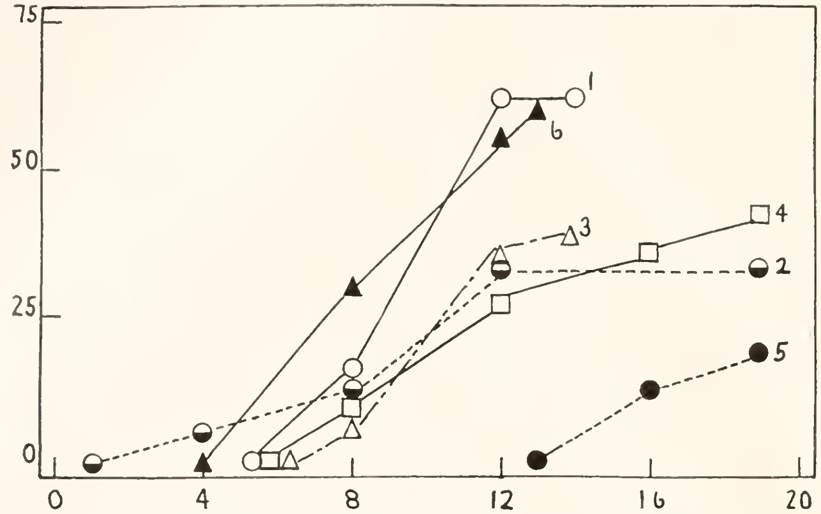

FIG. 3. Series B. Percentage of original population of *Calanus* attaining moulting stage when fed different concentrations of *Nitzschia*. Symbols as in Fig. 2. Ordinates: *Percentage of moults*. Abscissæ: *Time in days*.

If each *Calanus* captured daily the diatoms from 2.5 cc. of water, then in Experiment B4 in which some growth obviously took place, each individual obtained per day only 37,500 cells, $0.034\gamma N$, and between 8 and 16 per cent of the food requirement calculated from oxygen consumption. The higher value assumes all the nitrogenous matter to be utilized; the lower value is based on the assumption that one-half is utilized.

Discussion

The filtering rate of *Calanus* in these experiments was less than that found by Fuller and Clarke using carmine particles, the previous value at 13° C. being 5.61 cc. per day against 1.09 cc. reported above. As the carmine particles tended to settle and were more difficult to count, the present values are the more reliable. Harvey (private communication) has found filtering rates of from 50 to 100 cc. per day using the larger diatoms *Lauderia* and *Ditylium*, though his values are based on only a few experiments. It seems probable that large particles are captured more readily, though the carmine particles mentioned above were somewhat smaller than *Nitzschia*.

Whether these varying results represent actual differences in the rate of filtering is a moot point. Possibly the variance depends upon the efficiency with which the maxillary bristles strain out particles of different sizes and shapes. It may be that small cells, such as *Nitzschia*, pass between the bristles so that a copepod retains only a small percentage of the *Nitzschia* in the water current which it generates. This is the author's view. On the other hand some investigators believe that copepods possess other food-capturing mechanisms besides filtration.

Lowndes (1935), basing his opinion on anatomical grounds, and on observation of living unrestrained animals, states that feeding of the calanoid copepods cannot be merely automatic, non-selective filtering, but involves the finding and grasping of food particles. *Calanus* has two distinct types of movement: (1) saltation in which propulsion is furnished by the antennules and swimming feet, and (2) a slow glide in which the motive force comes from the vibration of the mouth parts. It is the latter which furnishes the feeding current. Often this species remains suspended quietly in the water for several minutes. Since the eye of copepods is not capable of forming images, recognizance of food material would have to be chemical or tactile in nature. In a turbulent fluid medium, only tactile stimuli would be serviceable in precisely locating food. Saltations might enable a copepod to try out different regions for food, the filtering mechanism being called into play only when food was encountered. One would thus expect more filtering where food was abundant since more particles would be encountered. On the other hand, the rate of feeding might be controlled by the amount of food ingested, so that filtering would slow down when food was abundant and speed up when food was scarce. No tendency towards a change in the filtering rate was shown. Harvey's results agree in this respect. Two conclusions are possible: (1) the rate of filtering is independent of the amount of food ingested or encountered; (2) even in the highest diatom concentrations used in these experiments there was insufficient food so that filtration was maintained at a maximal rate. Since in many experiments (e.g. 7 and 8) diatoms were present in concentrations much higher than are ever found in nature, alternative (2) is ruled out. The evidence definitely favors the theory that filtration is an automatic process independent of the concentration of food, though its rate is modified by physical factors in the environment.

These factors insofar as they have been studied may be considered in relation to the ecology of the species. The suggestion of a diurnal feeding rhythm is nicely correlated with the habit of vertical migration in this species which rises to the surface at night, and sinks below this rich food zone during the day. Neither phenomenon is absolutely regular. Probably both are dependent upon the physiological state of the animal which conditions its response to light (see Clarke, 1934). The ability of *Calanus* to feed at temperatures as low as 3° C. and its high rate of filtering at 8° indicate a physiological adaptation to life in the colder parts of the ocean.

The question of the food requirement of *Calanus* is perhaps of greatest fundamental importance, since the rôle of the food supply in the regulation of the *Calanus* population can only be determined when the minimum requirements for metabolism and growth are known. The discrepancy between the requirement calculated from oxygen consumption measurements and that estimated from measurements of the filtering rate and the concentration of particulate matter in the sea has been mentioned above.

It was hoped that the experiments of Series B reported herein would resolve this difficulty, but the heavy mortality occurring in them makes definite conclusions unsafe. However, a higher percentage of copepods attained the moulting stage in Experiment B4 with a minimum concentration of diatoms, than in Experiment B2 with ten times as many, although there was one successful moult in the latter experiment against none in the former. Presumably each copepod in Experiment B4 received not over one-sixteenth of the food requirement calculated from oxygen consumption. It is unlikely that the filtering rate in B4 was sixteen times greater than in any of the experiments in which the rate was measured. Thus it is probable that the minimum nutritive requirement for growth is less than previously believed, though it is as yet impossible to set a definite figure. Why higher concentrations of food have so little effect on the rate of growth is problematical unless the concentration in B4 itself is well over the required minimum. Another problem is the reason for the rapid growth of *Calanus* in harbor water. Possibly qualitative as well as quantitative differences in the food supply are involved in this case.

When larger particles serve as food the effective filtering rate is probably higher. If so, the concept of the feeding rate taken at the beginning of this work—namely the volume of water filtered per unit of time—must be modified. Instead the feeding rate must be considered as the number of particles of a given type captured per unit of time when present in a given concentration.

It follows that the investigation of the minimum concentration of food which will support life and growth in *Calanus* and in other species must employ several types of food organisms. The problem is a complex one, for it is possible that the metabolic rate of *Calanus* varies with the amount of food taken in as well as with other environmental factors.

SUMMARY

(1) The filtering rate of *Calanus finmarchicus* on the diatom, *Nitzschia closterium*, was measured under different conditions of diatom concentration, light and temperature, by observing the reduction in concentration of the diatoms. Change in diatom concentration had no effect on the rate of filtration. There was some evidence of a diurnal feeding rhythm with greater activity at night. Feeding was most rapid at 8° C., slower at 13° and 3°.

(2) Studies on the growth of *Calanus* supplied with different concentrations of diatoms indicated that the food requirement is less than has been calculated from oxygen consumption measurements. This is also borne out by comparing the amount of particulate organic matter in the sea with the rate of feeding measured in the laboratory.

(3) The view that the feeding activity of *Calanus* is automatic filtering is favored by these experiments.

(4) Approximately one-half the organic nitrogen content of *Nitzschia* is in a form available to *Calanus*.

(5) These results are correlated with the ecology of *Calanus* as studied in the field.

The author is indebted to Dr. George L. Clarke for many helpful suggestions, and to Miss Margaret Frothingham for technical assistance.

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CYST FORMATION IN THE GLOMERULAR TUFTS OF CERTAIN FISH KIDNEYS

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In an earlier study (Grafflin, 1933), the paradox, in an old specimen of daddy sculpin (*Myoxocephalus scorpius*), of a kidney showing many glomeruli anatomically but no glomerular function physiologically was satisfactorily explained. The glomeruli had been rendered incompetent by degenerative changes affecting both the vascular tufts and the neck segments, so that it was practically impossible to find a single glomerulus which on anatomical grounds could be considered functional. In young fish of the same species there was found adequate anatomical basis for the varying, but low, glomerular function which could be demonstrated physiologically. However, considerable degeneration was already present in the youngest specimens examined, and these changes became steadily more prominent with increasing age (as judged by weight). For a complete discussion of the glomerular changes noted, the original paper should be consulted. Some glomeruli, relatively quite infrequent, exhibited cystic cavities in their vascular tufts, and this is the particular problem which concerns us here.

> " Probably the most interesting glomeruli in this material are those showing what may be called a central cystic degeneration. In some instances the tuft shows a more or less spherical and very well-delimited cavity which is either entirely free from coagulum or shows it in only small amounts. These clear spaces show a wide variation in size. . . . In other tufts the cyst shows rather poor delimitation and considerable amounts of coagulum." (Grafflin, 1933, p. 65.)

At that time such cyst formation had not been observed in the glomeruli of any other species (fish or higher vertebrates). Although the specimens of *M. scorpius* showed rather numerous parasites in the kidney, all of the evidence indicated quite clearly that the parasitism played no rôle whatsoever in the observed glomerular changes. Under the circumstances, one would reasonably be led to the conclusion that the cysts represented one manifestation of the generalized process of glomerular degeneration. However, the following statement was made: " The sharp delimitation of these central cysts in some cases suggests that we may even be dealing here with a malformation of the tuft" (p. 66). In the course of the last several years instances of cyst formation have been found in the glomerular tufts of seven additional species, two of them lungfishes, four of them marine teleosts, and one of them an arid-living reptile (the horned toad). None of these species exhibits the generalized glomerular degeneration characteristic of *M. scorpius*. Further discussion of the problem will be deferred until after a description of the findings.

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[1] Fellow of the John Simon Guggenheim Memorial Foundation (1934). The specimens of *Ophichthys, Crenilabrus* and *Corvina* were collected at the Stazione Zoologica, Naples, Italy. I wish to thank Professor R. Dohrn for his many kindnesses while I was a guest in his laboratory.

LUNGFISHES

Protopterus æthiopicus (*African lungfish*)

In one of the available specimens (No. 21) two striking instances of cyst formation were observed, of widely different sizes and both exhibiting a delicate coagulum (Figs. 1 and 2). In another specimen (No. 34) two cysts were likewise observed (Fig. 3). Though closely adjacent, they are in different glomerular tufts, which are located, however, in the same glomerular cluster. In these latter instances the cyst contains much heavier coagulum, including what appears to be cellular debris, and in addition some well-formed cellular elements, presumably inwandering leucocytes. All four of these cysts are sharply delimited, and are lined by a flattened, endothelium-like epithelium (particularly well shown in Fig. 3). Immediately outside of this epithelial lining there is a well-defined basement membrane, which stands out quite clearly in Fig. 3. In comparison with non-cystic glomeruli, there is no increase in cellularity of the tuft, and no detectable abnormality of

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EXPLANATION FOR PLATE 1

FIGS. 1 and 2. A large and a small cyst in glomerular tufts of *Protopterus æthiopicus*. Iron hematoxylin and orange G.

FIG. 3. Two cysts, side by side, in adjacent glomerular tufts of *Protopterus æthiopicus*. Heidenhain-azan. × 410.

FIG. 4. Parasite in glomerular tuft of *Protopterus æthiopicus*. Iron hematoxylin and orange G.

FIG. 5. Small cyst in glomerular tuft of *Lepidosiren paradoxa*. Iron hematoxylin and orange G.

FIGS. 6, 7 and 8. Cysts in glomerular tufts of *Myoxocephalus octodecimspinosus*. Heidenhain-azan.

FIG. 9. Glomerular tuft of *Myoxocephalus octodecimspinosus*, showing extensive region of degenerative change, interpreted as probable precursor of cyst formation. Heidenhain-azan.

All sections 5 μ. All microphotographs at × 350 except Fig. 3. All cysts, except the small one to the left of Fig. 3, photographed at point of greatest cross-sectional area.

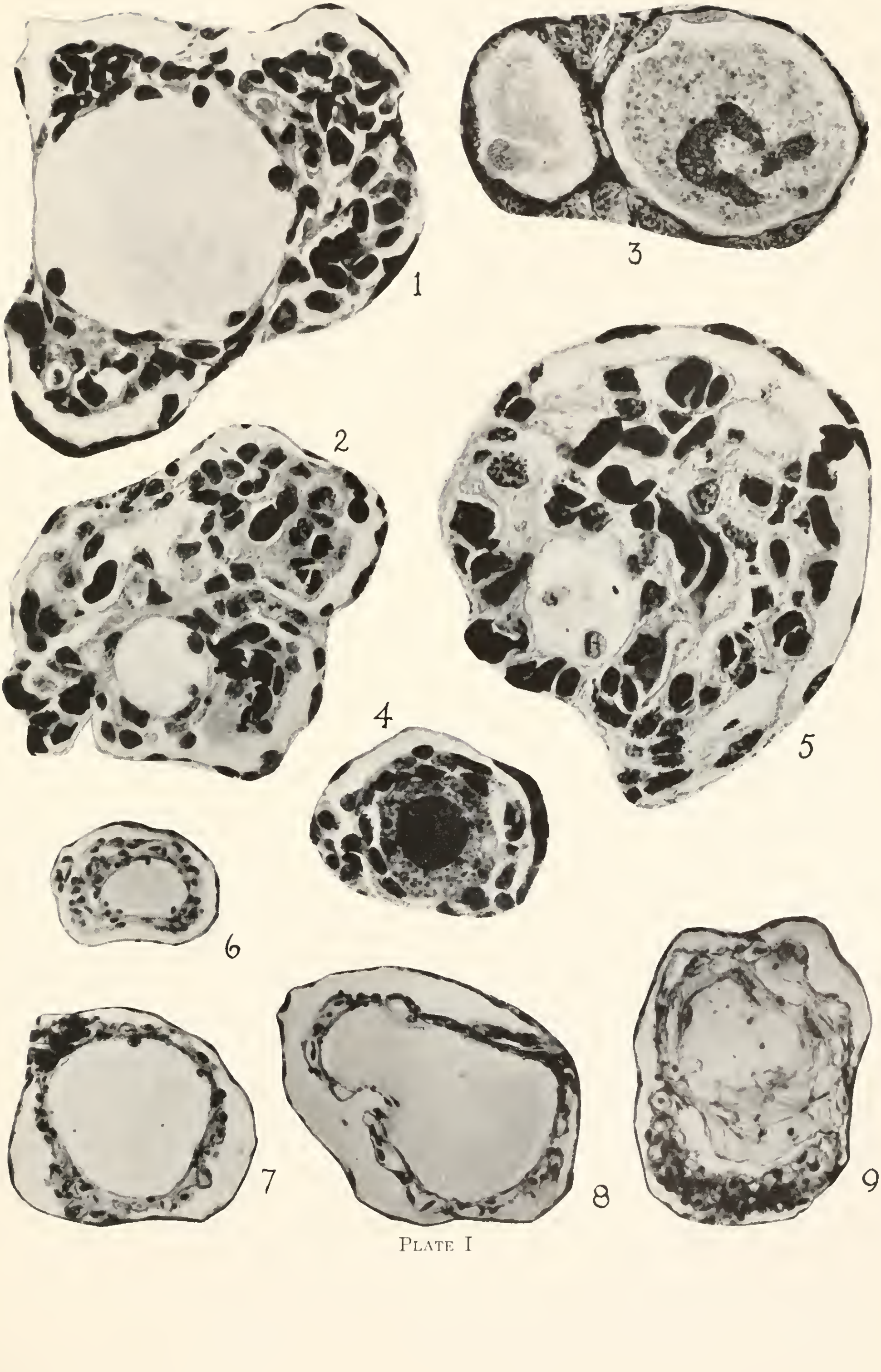

PLATE I

the afferent and efferent vessels or of the peripheral capillaries. The intracapsular spaces are free from coagulum, the ciliated neck segments are normal, and the first portions of the proximal convoluted segments are entirely comparable with those of adjacent nephrons showing no glomerular abnormality. In Specimen No. 21 the presence of a parasite in the glomerular tuft is a not infrequent finding. These parasites always exhibit a characteristic structure, which is shown in Fig. 4. Careful study of the material has failed to unearth any positive evidence that the parasites may ultimately be associated with the appearance of cysts such as those described above, but for the present such an association cannot be denied and must be left an open question.[2]

Lepidosiren paradoxa (*South American lungfish*)

In one of the specimens available a single small cyst was observed (Fig. 5). It is essentially similar in structure to the cyst shown in Fig. 2. It differs in having a less rigidly spherical outline and in containing, in addition to coagulum, a few formed cellular elements. The general statements made above concerning the cystic glomeruli of *Protopterus* likewise apply to this instance in *Lepidosiren*. Although parasitic remains are present in the kidney, they have never been observed in the glomeruli, and it is concluded that they have nothing to do with the formation of the cyst in question.

EXPLANATION FOR PLATE II

FIGS. 10, 11, 12, 13 and 14. Five instances of cyst formation in glomerular tufts of *Ophichthys imberbis*. 5 μ.

FIG. 15. Normal glomerular tuft of *Ophichthys imberbis*, without cyst formation, showing large intracapsular space frequently observed in the present material. 5 μ.

FIGS. 16 and 17. Isolated instances of cyst formation in glomerular tufts of *Crenilabrus pavo* (Fig. 16) and *Corvina nigra* (Fig. 17). 5 μ.

FIGS. 18 and 19. Two instances of cyst formation in glomerular tufts of the horned toad, *Phrynosoma cornutum*. 10 μ.

All sections stained with hematoxylin and eosin; all microphotographs at × 350; all cysts photographed at point of greatest cross-sectional area.

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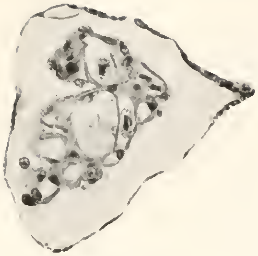
[2] These specimens were collected in Africa by Professor Homer W. Smith. For the sake of completeness the following data are given. Specimen No. 21: collected in July, 1928; kept alive in dry estivation from November 1, 1928, for 427 days; replaced in water for ten days and killed, as it seemed about to die after the appearance of superficial infection. Specimen No. 34: collected in July, 1928; kept in water until January 10, 1930, being fed regularly; accidentally killed by exposure to cold. From a study of the available material, there is no evidence that a period of estivation has any bearing whatsoever upon the formation of cysts.



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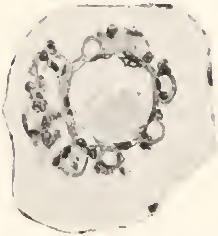

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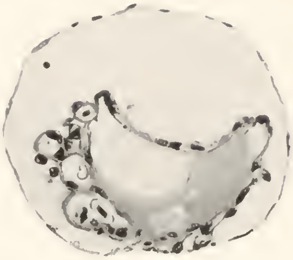

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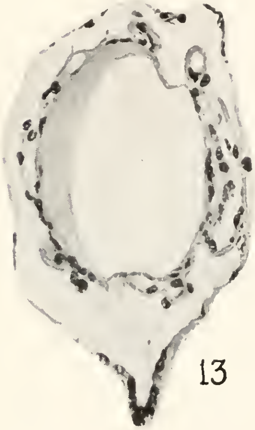

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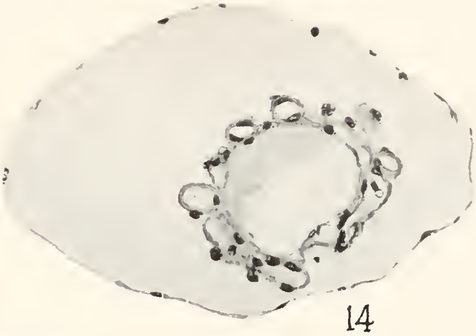

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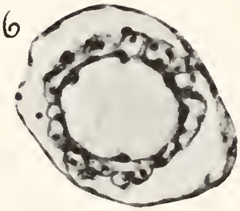

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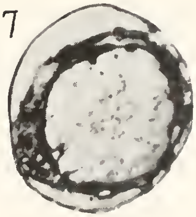

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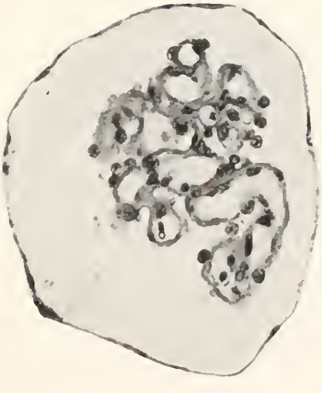

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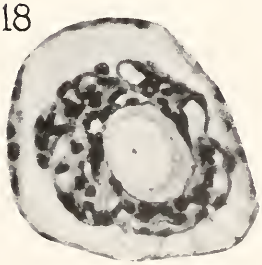

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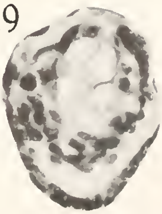

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Plate II

MARINE TELEOSTS

Myoxocephalus octodecimspinosus (sculpin)

As reported in detail elsewhere (Grafflin, 1937), the glomeruli of this species exhibit a wide variation in size and vascularity, but are in no sense the seat of generalized degenerative processes such as characterize the glomeruli of the closely related daddy sculpin (*M. scorpius*). Nevertheless, glomerular cysts have been found to occur in all of the six specimens carefully examined. In five of these specimens such cysts are present in only relatively small numbers. In the sixth, in one restricted portion of the kidney, cystic glomeruli comprise almost one-third of the total glomeruli. No reason for their large number in this region is apparent. Three examples of cystic glomeruli, to show the range in size encountered, are given in Figs. 6, 7 and 8. The cysts are sharply delimited, are lined by a markedly flattened epithelium (the nuclei of which are particularly apparent in Fig. 6), and contain coagulum of varying density. As in the lungfishes, the intracapsular spaces are free from coagulum, and the afferent and efferent vessels and the peripheral capillaries are apparently normal, as are the associated neck and proximal convoluted segments. A careful search for some lead as to the mechanism by which these cysts might arise yielded only the isolated glomerulus shown in Fig. 9, which is nevertheless very striking. While the lower part of the tuft retains essentially normal glomerular organization, the bulk of the tuft has lost it completely, and exhibits for the most part a coarse, stringy meshwork containing numerous naked nuclei. It seems reasonable to suppose that the degenerative process, which is here so clearly apparent, might eventually result in the formation of a large cyst of the type shown, for example, in Fig. 8. Small unicellular parasites regularly occur in the sculpin kidney. However, since they are never found in the glomeruli, it is concluded that they play no rôle in the formation of the cysts.

Ophichthys imberbis

In material from the caudal kidney of the single specimen available for study (Naples—weight 28 grams), five instances of cyst formation have been observed (Figs. 10–14). In all cases the ciliated neck segment is fully patent and entirely normal in appearance, and the intracapsular space shows no trace of coagulum. That the relatively large size of the intracapsular space (see particularly Fig. 14) bears no essential relationship to the presence of the cysts is clear from an examination of the normal glomeruli of this specimen, in which this space is frequently unusually large (Fig. 15). The cysts vary in size, but are all sharply delimited and are lined by a flattened epithelium. They contain a considerable amount of coagulum, which has withdrawn to one side of the cavity, presumably in the course of fixation. The capillaries persisting in the outer rim of the tuft are normal in appearance and fully patent, and the visceral epithelium is identical in thickness and appearance with that of the normal glomeruli. The afferent and efferent vessels are likewise normal. The cysts tend to be spherical in shape, but in one case (Fig. 12) the cyst wall has collapsed, perhaps in the course of fixation. The glomerulus shown in Fig. 12 is to be compared with one previously described in the daddy sculpin (Grafflin, 1933, Figs. 7 and 15).

In three instances (Figs. 11, 12 and 14) the ciliated neck can be readily traced into the first portion of the proximal convoluted segment, which is entirely normal in appearance. In another case (Fig. 10) the sections do not include the transition. In the fifth instance (Fig. 13) the ciliated neck, approximately 100 μ in length, passes into a curious segment with flattened cuboidal epithelium, which shows neither cilia nor brush border. The cytoplasm is scanty and lightly eosinophilic; the nuclei are closely packed but show no signs of degeneration. This segment persists for about 250 μ, at which point it shows a transition to the normal epithelium of the first portion of the proximal convoluted segment.

Crenilabrus pavo

In material from the caudal kidney of the single specimen available for study (Naples—weight 120 grams), a single instance of cyst formation was observed (Fig. 16). The cyst is spherical and sharply delimited, is lined by a flattened epithelium, and contains a moderate amount of coagulum. The peripheral capillaries and the afferent and efferent vessels are entirely normal in appearance, and the visceral epithelium is unthickened. There is a very delicate coagulum in the intracapsular space. The ciliated neck segment, which is very short, is fully patent, and the associated first portion of the proximal convoluted segment shows no abnormality.

Corvina nigra

The solitary instance of cyst formation observed in this species (single specimen, caudal kidney; Naples—575 grams) is illustrated in Fig. 17. The cyst is essentially spherical, is lined by a flattened epithelium, and exhibits a rather coarse, stringy coagulum. A few naked nuclei, fairly well preserved, are found scattered at random through the coagulum. The peripheral capillaries, few in number, contain normal red cells, and the afferent and efferent vessels show no detectable abnormality. The glomerular membrane is in many places appreciably thickened. The neck of the tubule is patent, though the lumen is quite small, and the first portion of the proximal convoluted segment is entirely normal in appearance.[3]

Horned Toad (Phrynosoma cornutum)

In surveying the available sections of the kidney of the horned toad, which is an arid-living reptile, two instances of cyst formation, entirely comparable with those observed in the fishes, were found (Figs. 18 and 19). Only a portion of the glomerulus shown in Fig. 18 is present in the sections, and both the afferent and efferent vessels and the neck segment are absent. The cyst is spherical and well-delimited, is lined by a flattened epithelium, and contains a considerable amount of coagulum. In the two sections adjacent to the one photographed there is present a dense, irregular, deeply basophilic mass of debris, which occupies perhaps one-quarter of the total cross-sectional area of the cyst. The peripheral capillary loops contain normal red cells, and show no thickening of the glomerular membrane as compared with the normal. There is a distinct coagulum in the intracapsular space. This glomerulus has formerly been briefly noted by Vilter (1935, p. 383). The appearance of the cyst in Fig. 19 is deceptive, due to the manner in which the coagulum has been precipitated. Actually the cyst is sharply delimited from the surrounding tissue, and is lined by flattened epithelium. The glomerular membrane is not thickened, and the peripheral capillary loops contain normal blood cells. The afferent and efferent vessels seem entirely normal, and the neck segment is fully patent.

Discussion

In seeking for an explanation for the formation of the cysts described above, we are led to the following considerations:

(1) All of the evidence indicates quite clearly that the presence of parasites in the kidney has no bearing upon the formation of cysts, except in the case of one specimen of *Protopterus* (No. 21). In this specimen, the tendency of the parasites to locate in the glomerular tuft is suggestive. The parasite might become walled off, and, with the sub-

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[3] In this specimen one small, degenerate avascular tuft was observed which showed a central cavity containing basophilic debris. The peripheral rim of tissue was hyalinized and almost structureless, still containing scattered nuclei and nuclear fragments. This cavity is in no sense typical of the cysts discussed here, and will not be further considered.

sequent evacuation, or degeneration and absorption, of the organism, cystic cavities of the type observed might persist. However, there is no direct evidence in the material at hand that such is the case. From the available evidence, it is concluded that the glomerular cysts are formed predominantly, or entirely, on some basis other than parasitism of the glomerular tuft. If parasitism can play a causative rôle, it is a completely separate process and of minor importance for the present problem.

(2) It seems almost certain that at least some of the cysts are formed as the result of a degenerative process in the glomerular tuft. In favor of this view are some of the pictures observed in the kidney of *Myoxocephalus scorpius* (Grafflin, 1933) and the striking glomerulus observed in *Myoxocephalus octodecimspinosus* (Fig. 9).

The two instances of cyst formation in glomerular tufts of the horned toad are particularly interesting. In the first place, this is the only species above the fishes in which such cysts have yet been recorded. In the second place, the reptilian glomerulus usually exhibits a central, avascular, cellular core, which, according to Regaud and Policard (1903) and Cordier (1928), is made up of connective tissue. Such a core is constantly present in the glomeruli of the horned toad (Marshall and Smith, 1930; Vilter, 1935). In the two glomeruli illustrated in Figs. 18 and 19, the cystic cavities occupy the region of the typical central cellular core, and replace it to such an extent that no characteristic portion of the core is any longer recognizable. One is led to wonder whether the cysts might not have arisen as the result of degeneration of the central avascular area. The irregular mass described above for the larger cyst (Fig. 18) is opaque and amorphous, and has all the appearance of calcified debris; it might be construed as the remains of the original core.

Let us now examine the available fish material in the light of these considerations for the horned toad. The glomerular tufts of *Crenilabrus* tend to be somewhat cellular, and one occasionally finds a central avascular core. The tufts of *Corvina* tend to be quite cellular, and it is not infrequent to find a typical central cellular core, entirely comparable with that seen in the horned toad and pigeon (see below). In *Myoxocephalus scorpius* (Grafflin, 1933) many tufts show a markedly cellular center, which may be entirely avascular. In an earlier study (Grafflin, 1929) it was shown that the relatively few glomerular structures present in the kidney of the adult goosefish (*Lophius piscatorius*) have lost all connection with renal tubules. The important fact for the present problem is that eight out of thirty-one of these

" pseudoglomeruli " which were carefully studied showed a central degeneration of the glomerular tuft. In seven of them the center of the tuft was hyaline, eosinophilic and entirely avascular, and showed a few scattered nuclei and nuclear fragments; in two of these seven this central area was vacuolated in addition.[4] In all of these tufts the central hyaline area was very sharply delimited from the peripheral tissue.

If our well-delimited cysts are to be interpreted in terms of degeneration and liquefaction of a central avascular portion of the tuft, certainly it is just as conceivable that such a process could occur in all of the fishes described above as in the horned toad. In favor of this interpretation are the amorphous mass in one of the cysts of the horned toad and the scattered nuclei in the cyst of *Corvina.*

However, ranged against such an interpretation are the following facts. (1) The two cysts observed in the horned toad are isolated instances, whereas one might reasonably expect them to be numerous on this basis. (2) In the pigeon, whose glomeruli likewise exhibit a cellular avascular core, an extensive search of abundant material failed to reveal a single instance of cyst formation (Vilter, 1935). (3) Though many glomeruli of the goosefish show marked degeneration of the central part of the tuft, no instance of cyst formation in such a tuft has yet been observed. (4) In the specimens of *Ophichthys, Lepidosiren* and *Protopterus,* the glomerular tufts are well vascularized, and no accumulations of cells have been observed which in any way suggest a cellular avascular core. Similarly in the sculpin (*M. octodecimspinosus*), in the usual range of glomerular size, no well-defined central core has yet been observed. (5) Some of the cysts are very small, and it seems perfectly clear that as we see them they are at their maximum size. It is hardly conceivable, in view of the findings in the pigeon, that an avascular region of the size represented by these small cysts would undergo degeneration. (6) The cysts are in general spherical, and give every indication of having contained fluid under pressure. If we were dealing merely with a degeneration of the central portion of the tuft, one would more logically expect collapse of the tuft rather than distension of the type observed.

In summary, while some of the glomerular cysts are apparently formed on the basis of a degenerative process in the glomerular tuft, it seems unlikely that all of the cysts which we have observed could arise in this manner.

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[4] The eighth tuft was very small and atrophic, and showed a central degeneration to the point of cavity formation, the cavity containing granular debris. The picture is not at all typical of the cysts discussed here, and will not be further considered.

In the legend to Fig. 7 of this earlier paper there is an obvious error. It is clear from the illustration that no ciliated neck segment is present, and that the intracapsular space opens directly into a segment whose cells exhibit the brush border characteristic of the epithelium of the proximal convoluted segment.

(3) It is suggested that many of the glomerular cysts herein described probably result from a malformation of the glomerular tuft. The cyst might be laid down early in embryological development, or it might be formed rather late, when glomerular organization is already well advanced. In the latter case two possibilities immediately suggest themselves: (*a*) the walling-off of a deep crevice between adjacent lobulations of the tuft; (*b*) the occlusion at both ends of a portion of one of the glomerular capillaries. On either basis one could readily understand (1) the flattened epithelial lining of the cyst; (2) the subsequent enlargement of the cyst without, at the same time, any encroachment upon or collapse of the surrounding capillaries; and (3) the presence within the cyst of coagulum, which would represent simply a seepage of plasma proteins into the completely closed cavity. Also, such a mode of formation would be consistent with the small size of some of the cysts and the failure to find, in adult animals, more than occasional suggestive intermediate stages in cyst formation.

SUMMARY

Well-delimited glomerular cysts have been observed in the kidneys of the following species of fishes: *Protopterus æthiopicus, Lepidosiren paradoxa, Myoxocephalus scorpius, Myoxocephalus octodecimspinosus, Ophichthys imberbis, Crenilabrus pavo, Corvina nigra;* and in the horned toad, *Phrynosoma cornutum.* It is concluded that these cysts are probably formed in two ways: (1) on the basis of a degenerative process in the glomerular tuft; (2) as the result of a malformation of the glomerulus in embryological development.

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THE PRODUCTION OF INTERMEDIATE-WINGED APHIDS WITH SPECIAL REFERENCE TO THE PROBLEM OF EMBRYONIC DETERMINATION [1]

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Aphids which are intermediate in structure between the parthenogenetic and the gamic type have already been studied in relation to the order of embryonic segregation (Shull, 1930, 1931, 1933). More abundant and more easily produced at will are those intermediates which are structurally between the winged and wingless parthenogenetic types. They offer the same opportunity to examine certain features of the mechanism of development as do any other intermediates which can be artificially produced.

Partly to obtain large numbers of these winged-wingless intermediates for analysis, and partly in the hope that the method of obtaining them would throw some light on their nature, extensive experiments in which the environmental agents known or believed to modify the aphid cycle were combined in a variety of ways were carried out over a period of years. This paper describes the experiments, which involved a total of 530,133 aphids, of which 9,152 were intermediate-winged.

The Stocks of Aphids

Two independent strains of aphids of the species *Macrosiphum solanifolii* have been used. One was collected near Ann Arbor in the year 1923 and has been maintained parthenogenetically ever since. This strain was clone A of a former paper (Shull, 1932) until it experienced a "mutation" in the fall of 1929, in which its characteristics were greatly altered, becoming clone A′ of that paper. The other strain was collected in Ann Arbor in 1931; to date none of the results from this line has been published.

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[1] Contribution from the Department of Zoölogy, University of Michigan.

This investigation is part of a series which has been aided over a period of years by grants from the American Academy of Arts and Sciences, the National Research Council, and the Elizabeth Thompson Science Fund, as well as from the Faculty Research Fund of the University of Michigan.

Since it has been shown that conditions affecting the aphids at least a generation in advance of the beginning of an experiment may modify the results of the experiment (Shull, 1935), four different stocks of each of the two strains were continuously reared. One was kept at a temperature of 24°, one at 14°, one at alternating temperatures (24° for 8 hours, 14° for 16 hours daily), and one at room temperature which fluctuated considerably. All were in continuous light which was not necessarily of uniform intensity.

The Method of the Experiments

In general a complete set of experiments would proceed as follows. From each of the eight stocks mentioned above a number of aphids, both winged and wingless, were drawn shortly before (or in a few cases just after) they became adult. Some of each type of female were placed at a temperature of 24°, others at 14°. In each of these temperatures some were kept in continuous light, others in intermittent light (8 hours light, 16 hours darkness). In addition, some aphids from each of the stocks except the one in room conditions were brought to room temperature for one generation prior to the beginning of the experiment; their offspring, having grown up at room temperature, were then reared in each of the various combinations of conditions just outlined.

It was never possible to do all of these things at once. However, all experiments involving one stock, and sometimes those from two of the stocks, could be done simultaneously. Since fairly consistent results were obtained from each treatment, it is unlikely that any serious error has been introduced by performing the various parts of an experiment at different times.

Effect of Continuous and Intermittent Light in the 1923 Strain

The results of these experiments can be presented only as total numbers of individuals of the several kinds produced under each combination of conditions. This is best done in tabular form. In branching tables, the contrasting conditions placed nearest the data are the ones whose effect is best shown, since pairs of adjoining numbers represent the contrast. Other contrasts are between numbers at some distance from one another, and are less readily seen. It will be necessary, therefore, to arrange the data in several ways to show the significant results.

In Table I the last column preceding the data (the fourth column) includes the two contrasted light conditions, namely, continuous and intermittent (8 hours of light, 16 hours of darkness). It is therefore the effect of light which is best shown by this table. To save space the

Table I

The percentages of winged and intermediate-winged offspring produced by both wingless and winged aphids of the 1923 strain in various combinations of light and temperature both during and before the experiments.

Stock parents came from	Temperature of parents preceding experiment	Temperature of parents during experiment	Light on parents during experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
24°	24°	24°	Cont.	7,640	50.7	0.26	12,816	59.7	0.37
			8–16	5,832	32.5	3.23	6,777	37.0	4.01
		14°	Cont.	7,835	46.5	0.18	17,056	42.4	0.28
			8–16	5,592	16.6	5.17	17,335	19.2	6.35
	Room	24°	Cont.	2,522	47.4	0.12	2,261	40.0	0.04
			8–16	1,650	38.4	3.03	1,737	28.6	4.38
		14°	Cont.	2,387	62.7	0.08	2,301	32.2	0.22
			8–16	2,445	15.4	4.05	1,647	9.0	10.75
14°	14°	24°	Cont.	6,550	49.4	0.11	8,309	33.6	0.13
			8–16	5,240	47.4	5.42	7,085	20.5	2.87
		14°	Cont.	7,182	55.0	0.07	6,806	21.3	0.13
			8–16	5,307	20.4	8.95	4,084	11.5	11.75
	Room	24°	Cont.	880	51.1	0.11	3,329	42.4	0.24
			8–16	846	39.7	5.52	3,326	29.8	2.56
		14°	Cont.	1,238	50.8	0.00	3,630	29.6	0.08
			8–16	1,017	18.6	7.37	3,169	10.4	9.09
Alt.	Alt.	24°	Cont.	5,581	49.3	0.13	10,505	55.3	0.39
			8–16	5,547	39.5	2.94	9,205	43.7	4.20
		14°	Cont.	7,257	35.8	0.12	12,786	39.6	0.13
			8–16	5,702	13.0	7.87	8,975	12.9	10.92
	Room	24°	Cont.	2,902	44.9	0.21	4,094	35.8	0.07
			8–16	2,709	35.4	3.03	3,545	25.3	2.03
		14°	Cont.	3,225	44.6	0.09	4,650	34.1	0.04
			8–16	2,760	17.8	8.48	3,190	7.4	7.62
Room	Room	24°	Cont.	4,415	46.8	0.18	8,015	45.8	0.29
			8–16	3,912	41.1	2.94	6,821	30.3	4.31
		14°	Cont.	4,694	43.8	0.17	9,268	44.8	0.21
			8–16	3,049	13.9	5.77	7,263	10.8	9.54

actual numbers of individuals of the several kinds are not given; only their percentages of the total, which are essential to quick comparison, are indicated. Any one who wishes to know the numbers can readily calculate them from the total. Though this paper is concerned only with intermediate individuals, the percentages of normal winged aphids is also given for the sake of a comparison to be made on a later page.

From Table I it is clear that more intermediate-winged aphids of this strain were produced in intermittent light than in continuous. This is true for both wingless and winged parents, for every stock, and for all temperatures both before and during the experiments. That is, in every combination of other agents, intermittent light yielded more intermediates than did continuous light. Moreover, the differences are large ones; in no instance is the ratio less than 10:1, and in many sets of conditions the intermediates were 20 or even 50 times as numerous in intermittent as in continuous light. Evidently continuity or discontinuity of light is of major importance in the production of intermediates in the 1923 strain.

Effect of Current Temperature in the 1923 Strain

The results of the experiments with the 1923 strain are rearranged in Table II so as to put the temperatures used during the experiment (24° and 14°) in the fourth column, next to the data, thereby contrasting most sharply the effects of these current temperatures.

The last column of this table, relating to those reared in intermittent light, shows the most striking contrast. In every combination of other conditions, more intermediate-winged offspring were produced at 14° than at 24°. The differences are fairly large, the ratios ranging roughly from 1.3:1 up to nearly 4:1. These differences are obtained, however, only in intermittent light.

In continuous light (column 7), the differences are small and mostly in the opposite direction. That is, more intermediates are produced at 24° than at 14° (one exception). Though all the numbers are small, the fact that nearly all the differences are of the same sign can hardly mean other than that there is a significant reversal of the effect of temperature in continuous light as compared with the temperature effect in intermittent light.

Wingless and Winged Parents in the 1923 Strain

By rearranging the experiments with the two types of parents at the right of the branching table, as is done in Table III, whatever difference there is in the tendency of wingless and winged parents to produce intermediate-winged offspring is best shown.

TABLE II

The data of Table I rearranged to show the contrast between 24° and 14° temperature in the production of intermediate-winged aphids by the 1923 strain.

Nature of parents	Stock parents came from	Temperature of parents preceding experiment	Temperature of parents during experiment	Offspring					
				Produced in continuous light			Produced in 8-hr. light, 16-hr. darkness		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
Wingless	24°	24°	24°	7,640	50.7	0.26	5,832	32.5	3.23
			14°	7,835	46.5	0.18	5,592	16.6	5.17
		Room	24°	2,522	47.4	0.12	1,650	38.4	3.03
			14°	2,387	62.7	0.08	2,445	15.4	4.05
	14°	14°	24°	6,550	49.4	0.11	5,240	47.4	5.42
			14°	7,182	55.0	0.07	5,307	20.4	8.95
		Room	24°	880	51.1	0.11	846	39.7	5.52
			14°	1,238	50.8	0.00	1,017	18.6	7.37
	Alt.	Alt.	24°	5,581	49.3	0.13	5,547	39.5	2.94
			14°	7,257	35.8	0.12	5,702	13.0	7.87
		Room	24°	2,902	44.9	0.21	2,709	35.4	3.03
			14°	3,225	44.6	0.09	2,760	17.8	8.48
	Room	Room	24°	4,415	46.8	0.18	3,912	41.1	2.94
			14°	4,694	43.8	0.17	3,049	13.9	5.77
Winged	24°	24°	24°	12,816	59.7	0.37	6,777	37.0	4.01
			14°	17,056	42.4	0.28	17,335	19.2	6.35
		Room	24°	2,261	40.0	0.04	1,737	28.6	4.38
			14°	2,301	32.2	0.22	1,647	9.0	10.75
	14°	14°	24°	8,309	33.6	0.13	7,085	20.5	2.87
			14°	6,806	21.3	0.13	4,084	11.5	11.75
		Room	24°	3,329	42.4	0.24	3,326	29.8	2.56
			14°	3,630	29.6	0.08	3,169	10.4	9.09
	Alt.	Alt.	24°	10,505	55.3	0.39	9,205	43.7	4.20
			14°	12,786	39.6	0.13	8,975	12.9	10.92
		Room	24°	4,094	35.8	0.07	3,545	25.3	2.03
			14°	4,650	34.1	0.04	3,190	7.4	7.62
	Room	Room	24°	8,015	45.8	0.29	6,821	30.3	4.31
			14°	9,268	44.8	0.21	7,263	10.8	9.54

This difference, though sometimes striking, is not everywhere of the same sign. In 21 of the 28 sets of conditions the winged parents produced the more intermediate offspring, and most of the differences are of some size. Of the 7 exceptions to this rule, 6 are from parents which were reared at room temperature throughout their immature stages, suggesting that perhaps the peculiarly variable conditions thus introduced were favorable to intermediate-winged offspring. However, the seventh exception (which is the seventh pair in the left side of Table III) was exhibited by parents not reared at room temperature; and the difference shown here is one of the more striking ones in the table. It seems impossible to bring the wingless and winged aphids under any general rule concerning their tendency to produce intermediate-winged offspring.

Effect of Antecedent Temperatures in 1923 Strain

Two other rearrangements of the results of the experiments would be required to show most directly the effects of the temperatures applied to the aphids prior to the beginning of the experiments. One table would show any difference in the number of intermediate offspring produced by aphids taken from the several temperature stocks, that is, the stocks reared at 24°, 14°, alternating, and room temperatures respectively. The other rearrangement would show whether bringing the parents to room temperature through all their immature stages before using them in an experiment affected the number of their intermediate-winged offspring. The differences which these tables would show are not regular enough to warrant their presentation in that form. A statement of them, however, should be made.

Keeping the experimental parents at room temperature through their immature stages, when that represented a change from previous conditions (as it would for aphids taken from the 24°, 14°, and alternating-temperature stocks), caused them to produce fewer intermediate-winged offspring in 16 out of 24 combinations of other conditions. Of the 8 which showed the opposite effects, 6 were reared at 24° during the experiment, the other 2 were reared at 14°. Among these 8 likewise were aphids from each of the four temperature stocks. Again it is impossible to state a general rule describing the effect of a one-generation transfer to room temperature in advance of an experiment.

Still less regular is the difference between the various temperature stocks, in their tendency to produce intermediate-winged offspring. Aphids from each stock yielded more intermediates than did those from all the other stocks, in from one to three of the combinations of other conditions. This is true regardless of whether the aphids were brought to room temperature for one generation before the experiment or not. Aphids changed from the alternating-temperature stock to room temperature yielded more than did those reared continuously at room temperature in some combinations of conditions, but fewer in other combinations.

TABLE III

Differences between wingless and winged parents of the 1923 strain in their production of intermediate-winged offspring under various conditions. (*ws* = wingless, *wd* = winged.)

Temperature of parents preceding experiment	Light on parents during experiments	Temperature of parents during experiments	Stock parents came from	Nature of parents	Total number of offspring	Percentage of offspring winged	Percentage of offspring intermediate-winged	Temperature of parents preceding experiment	Light on parents during experiments	Temperature of parents during experiments	Stock parents came from	Nature of parents	Total number of offspring	Percentage of offspring winged	Percentage of offspring intermediate-winged
24°	Cont.	24°	24°	*ws*	7,640	50.7	0.26	Room	Cont.	24°	24°	*ws*	2,522	47.4	0.12
				wd	12,816	59.7	0.37					*wd*	2,261	40.0	0.04
		14°	24°	*ws*	7,835	46.5	0.18				14°	*ws*	880	51.1	0.11
				wd	17,056	42.4	0.28					*wd*	3,329	42.4	0.24
	8–16	24°	24°	*ws*	5,832	32.5	3.23				Alt.	*ws*	2,902	44.9	0.21
				wd	6,777	37.0	4.01					*wd*	4,094	35.8	0.07
		14°	24°	*ws*	5,592	16.6	5.17				Room	*ws*	4,415	46.8	0.18
				wd	17,335	19.2	6.35					*wd*	8,015	45.8	0.29
										14°	24°	*ws*	2,387	62.7	0.08
												wd	2,301	32.2	0.22
											14°	*ws*	1,238	50.8	0.00
												wd	3,630	29.6	0.08
14°	Cont.	24°	14°	*ws*	6,550	49.4	0.11				Alt.	*ws*	3,225	44.6	0.09
				wd	8,309	33.6	0.13					*wd*	4,650	34.1	0.04
		14°	14°	*ws*	7,182	55.0	0.07				Room	*ws*	4,694	43.8	0.17
				wd	6,806	21.3	0.13					*wd*	9,268	44.8	0.21
	8–16	24°	14°	*ws*	5,240	47.4	5.42		8–16	24°	24°	*ws*	1,650	38.4	3.03
				wd	7,085	20.5	2.87					*wd*	1,737	28.6	4.38
		14°	14°	*ws*	5,307	20.4	8.95				14°	*ws*	846	39.7	5.52
				wd	4,084	11.5	11.75					*wd*	3,326	29.8	2.56
											Alt.	*ws*	2,709	35.4	3.03
												wd	3,545	25.3	2.03
											Room	*ws*	3,912	41.1	2.94
												wd	6,821	30.3	4.31
Alt.	Cont.	24°	Alt.	*ws*	5,581	49.3	0.13			14°	24°	*ws*	2,445	15.4	4.05
				wd	10,505	55.3	0.39					*wd*	1,647	9.0	10.75
		14°	Alt.	*ws*	7,257	35.8	0.12				14°	*ws*	1,017	18.6	7.37
				wd	12,786	39.6	0.13					*wd*	3,169	10.4	9.09
	8–16	24°	Alt.	*ws*	5,547	39.5	2.94				Alt.	*ws*	2,760	17.8	8.48
				wd	9,205	43.7	4.20					*wd*	3,190	7.4	7.62
		14°	Alt.	*ws*	5,702	13.0	7.87				Room	*ws*	3,049	13.9	5.77
				wd	8,975	12.9	10.92					*wd*	7,263	10.8	9.54

Antecedent temperatures on the whole thus give mixed results in the production of intermediate-winged offspring. All these relations can be made out from the tables already given, though the numbers contrasted are not in adjoining lines. It seems not worth while to bring them thus together in view of the impossibility of generalizing from them.

Wing Production and Intermediate Wings in the 1923 Strain

It is worthy of note that in general the greatest production of intermediate wings does not accompany the greatest production of wings. Intermittent light, with every combination of the other factors, resulted in more intermediate-winged but fewer winged individuals, than did continuous light (Table I). Current temperature of 14°, with intermittent light, produced likewise more intermediate-winged but fewer winged offspring than did 24°, in every combination of other factors (Table II, right). However, current temperature of 14°, with continuous light, produced mostly fewer intermediate-winged and at the same time mostly fewer winged offspring than did 24° (one or two exceptions, Table II, left half). The differences here are mostly small, however, for both the winged and the intermediate-winged.

Within any one of these major groups, the relation between wing production and intermediate wings may also be determined from their correlation. This has been calculated for the two halves of Table II separately. For the right half of that table, where production of intermediate wings is everywhere high and that of normal wings moderate, the correlation between the two is found to be $r = -0.68 \pm 0.10$. This means that in general the fewer the wings the more numerous the intermediate wings. This negative relation is not due, however, to a mere conversion of wings into half-grown wings at low temperature. Even if all the intermediate-winged aphids were converted into winged ones, there would still be in most conditions a considerable deficit of winged individuals at the lower temperature.

For the left half of Table II the corresponding correlation between wings and intermediate wings is $r = 0.26 \pm 0.18$, which is positive but not of proven significance. There is no simple relation, therefore, between production of wings and production of intermediates; but on the whole these two things are oppositely affected by the same sets of conditions.

The bearing of these facts on the nature of intermediates is discussed in a later section.

The 1931 Strain of Aphids

The 1931 strain of aphids differs very considerably from the 1923 strain in the production of both winged and intermediate-winged individuals. The proportion of intermediate aphids in the 1931 strain is only about one-eleventh of that in the 1923 line. In wing production, the 1931 strain resembles the 1923 strain during the latter's first six years; that is, before its "mutation" of 1929. The 1931 line is thus more like clone A than like clone A′ of a former paper (Shull, 1932). Both clone A and the 1931 strain produced more winged individuals in intermittent than in continuous light; and when the relatively weak light used in the recent experiments is taken into account, the degree of their response to light is probably not very different.

Effect of Continuous and Intermittent Light on Intermediates in the 1931 Strain

The experiments with the 1931 strain are first presented with the contrast between continuous and intermittent light most plainly shown, as in Table IV. In 27 of the 28 pairs of percentages of intermediate-winged offspring the larger percentage is that obtained in intermittent light. The numbers are mostly small, but the fact that in all but one pair the differences are in the same direction indicates the significance of the result.

The only situation in which the difference is very marked is that involving a succession of 24°, room, and 14° temperatures; that is, in which aphids from the 24° stock were put at room temperature where their offspring grew to maturity, and these offspring became the parents in the experiment at 14°. Both wingless and winged parents responded in the same decisive way, hence the large number of intermediate offspring can hardly be accidental.

The one exception to the direction of difference arose from a situation like that above except that aphids from the 14° stock were used. The preceding temperature (room) and the current temperature (14°) were the same. The difference was small, and the reversal of its sign is found only among the offspring of winged parents; hence it is of questionable meaning.

Effect of Antecedent Temperatures on Intermediates in the 1931 Clone

By rearranging the results obtained with the 1931 strain as in Table V, the effect of temperatures applied to the ancestors of the ex-

TABLE IV

The percentage of winged and intermediate-winged offspring produced by both wingless and winged aphids of the 1931 strain in different combinations of light and temperature both during and before the experiments.

Temperature of parents during experiment	Stock parents came from	Temperature of parents preceding experiment	Light on parents during experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
24°	24°	24°	Cont.	6238	11.5	0.06	6332	6.7	0.13
			8–16	5827	26.8	0.46	5055	18.8	0.69
		Room	Cont.	2094	32.8	0.00	1777	20.8	0.06
			8–16	2063	29.3	0.58	1659	25.8	0.60
	14°	14°	Cont.	6742	4.8	0.06	3411	1.0	0.09
			8–16	6169	33.9	0.24	3315	20.2	0.72
		Room	Cont.	2758	11.5	0.11	1824	4.6	0.00
			8–16	2913	32.4	0.27	1707	17.3	0.06
	Alt.	Alt.	Cont.	3720	7.2	0.05	5304	7.9	0.23
			8–16	3642	10.2	0.14	5144	22.5	0.45
		Room	Cont.	3945	12.4	0.03	1412	3.0	0.00
			8–16	2546	26.8	0.31	980	7.4	0.20
	Room	Room	Cont.	6709	21.0	0.18	6480	7.0	0.23
			8–16	5231	33.5	0.98	5214	18.4	0.98
14°	24°	24°	Cont.	6306	14.7	0.08	6587	10.7	0.08
			8–16	5187	75.7	0.42	5506	75.8	0.64
		Room	Cont.	2593	43.0	0.12	2086	39.8	0.10
			8–16	2196	53.6	2.46	1557	37.9	4.82
	14°	14°	Cont.	7356	9.4	0.05	3990	3.0	0.00
			8–16	6074	82.6	0.36	3381	67.7	0.62
		Room	Cont.	2874	12.6	0.14	1586	9.6	0.06
			8–16	2473	85.5	0.36	1712	84.0	0.00
	Alt.	Alt.	Cont.	4358	13.6	0.02	5735	3.1	0.04
			8–16	3774	89.1	0.19	5088	83.7	0.35
		Room	Cont.	4024	16.4	0.05	1441	3.3	0.00
			8–16	3134	86.8	0.10	1179	84.8	0.08
	Room	Room	Cont.	6463	14.6	0.11	6308	10.1	0.32
			8–16	6041	80.6	0.53	5012	75.1	1.06

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TABLE V

The data of Table IV rearranged to show the effect of different antecedent temperatures applied to the parent aphids of the 1931 clone.

Light on parents during experiment	Temperature of parents during experiment	Stock parents came from	Temperature of parents preceding experiment	Offspring					
				From wingless parents			From winged parents		
				Total number of offspring	Percentage winged	Percentage intermediate-winged	Total number of offspring	Percentage winged	Percentage intermediate-winged
Cont.	24°	24°	24°	6238	11.5	0.06	6332	6.7	0.13
			Room	2094	32.8	0.00	1777	20.8	0.16
		14°	14°	6742	4.8	0.06	3411	1.0	0.09
			Room	2748	11.5	0.11	1824	4.6	0.00
		Alt.	Alt.	3720	7.2	0.05	5304	7.9	0.23
			Room	3945	12.4	0.03	1412	3.0	0.00
		Room	Room	6709	21.0	0.18	6480	7.0	0.23
	14°	24°	24°	6306	14.7	0.08	6587	10.7	0.08
			Room	2593	43.0	0.12	2086	39.8	0.10
		14°	14°	7356	9.4	0.05	3990	3.0	0.00
			Room	2874	12.6	0.14	1586	9.6	0.06
		Alt.	Alt.	4358	13.6	0.02	5735	3.1	0.04
			Room	4024	16.4	0.05	1441	3.3	0.00
		Room	Room	6463	14.6	0.11	6308	10.1	0.32
8–16	24°	24°	24°	5827	26.8	0.46	5055	18.8	0.69
			Room	2063	29.3	0.58	1659	25.8	0.60
		14°	14°	6169	33.9	0.24	3315	20.2	0.72
			Room	2913	32.4	0.27	1707	17.3	0.06
		Alt.	Alt.	3642	10.2	0.14	5144	22.5	0.45
			Room	2546	26.8	0.31	980	7.4	0.20
		Room	Room	5231	33.5	0.98	5214	18.4	0.98
	14°	24°	24°	5187	75.7	0.42	5506	75.8	0.64
			Room	2196	53.6	2.46	1557	37.9	4.82
		14°	14°	6074	82.6	0.36	3381	67.7	0.62
			Room	2473	85.5	0.36	1712	84.0	0.00
		Alt.	Alt.	3774	89.1	0.19	5088	83.7	0.35
			Room	3134	86.8	0.10	1179	84.8	0.08
		Room	Room	6041	80.6	0.53	5012	75.1	1.06

perimental aphids is shown. The last column preceding the actual data shows the temperature applied to just one generation prior to the beginning of the experiments. Since, however, there is some coupling of the temperature applied to that generation with the temperature of preceding generations (not all combinations having been tried), it is a combination of the last two (third and fourth) columns of the branching part of the table to which the data are most closely related.

This arrangement shows that aphids taken from the room temperature stock yielded more intermediate-winged offspring than did those of any of the stocks kept at other temperatures (24°, 14°, alternating), provided the stock conditions were maintained up to the very beginning of the experiment. This is true of both wingless and winged parents, and of every combination of other conditions, though one of the differences is so slight as not to appear as a difference in the table.

Changing the parents to room temperature from one of the other temperatures (24°, 14°, or alternating) yielded on the whole more intermediate-winged offspring than did continuing the stock temperature up to the beginning of the experiments, if wingless parents were used, but fewer intermediates if winged parents were used. There are, however, three exceptions out of twelve to each part of that statement. Moreover, one of the exceptions is one of the only two very striking differences to which attention was called in the preceding section. These exceptions, which do not themselves possess any one characteristic in common, weaken the generalization that seems otherwise warranted.

Effect of Temperature and of Nature of Parents on Production of Intermediates in 1931 Strain

The remaining factors produce too little or too variable an effect on intermediacy to warrant rearrangement of the data in tables to show those effects clearly. The following statements of these irregular effects can be verified from the tables already given, by comparing percentages not adjacent to one another.

The nature of the parents has no regular influence on the number of intermediates. Winged parents produced more intermediate offspring in 17 combinations of external conditions, but fewer in 11 combinations. Eight of the latter group of 11 came from 14° and alternating temperature stocks and in addition were at room temperature for one generation before the experiment started.

Current temperature had no consistent effect, since aphids at 14° produced more intermediates than did those at 24° in 13 combinations, but fewer in 14, and an equal number in 1. No single factor is common to all of the 13, nor to all of the 14.

Relation of Intermediacy to Wing Production in the 1931 Strain

As was stated earlier, the only situation in which the 1931 clone produced very many intermediate-winged individuals was intermittent light and a current temperature of 14°, applied to parents which came from the 24° stock but which were reared at room temperature throughout their own immature stages (eighteenth line, Table IV).

These conditions are not exceptionally conducive to production of normal-winged aphids. Winged individuals were, it is true, more abundant (47 per cent) under these conditions than in the experiments as a whole (34 per cent) in the 1931 strain. But other combinations of conditions resembling this one yielded higher wing production as often as lower. Thus, changing only the current light from intermittent to continuous served to reduce wing production 13 per cent; and changing only the current temperature from 14° to 24° reduced wing production 19 per cent. But omitting only the change to room temperature during the immature stages of the parents in the experiments increased wing production 29 per cent; and taking the parents from the 14° or the alternating temperature stock instead of the 24° stock increased wing production 38 per cent and 39 per cent respectively. None of these percentages appears directly in any of the tables, since they are based on the combined output of both wingless and winged parents.

It may also be pointed out that the set of conditions yielding the most intermediate individuals produced fewer winged ones than any other set of conditions that included a current temperature of 14°, but more than any set involving 24° temperature during the experiments.

On the whole, therefore, the greatest number of intermediate aphids would seem to be produced under circumstances which favored medium wing production in this strain.

Less connection between wings and intermediate wings is to be seen in a comparison of lines 17 and 18 in Table IV. The two very large percentages of intermediates are shown in line 18. The conditions resulting in the aphids of line 17 differ from those producing the aphids of line 18 only in that they included continuous light instead of intermittent light. This one change reduced the proportion of winged aphids only moderately, but reduced the intermediates very greatly. Continuous light reduces the number of winged aphids in some other clones (clone A, for example, Shull, 1932), and in general does so for the 1931 strain used in these experiments. That response is well shown in Table V, where the percentages of winged individuals are consistently lower in the upper half of the table than in the lower. But in that upper half, the percentages in the ninth line stand out as far higher than any others. Continuous light had little of its usual effect on the number of winged offspring in this particular combination of other agents, but reduced the intermediate-winged individuals to as low a proportion as the average of all others in continuous light (upper half of Table V). It is possible, therefore, to change the production of intermediates without greatly changing the production of normal-winged aphids.

If the relation of intermediates to winged aphids is to be studied by the correlation method, the data of the 1931 strain must be broken up into three parts. Since intermittent light favors both wings and intermediate wings, strong correlation between the two would be shown by the data as a whole, but this would indicate nothing more than Table IV does. Current temperature also has a marked effect on wing production in intermittent light, though not in continuous light. Since calculation of correlation coefficients is justified only when the two variable qualities are distributed unimodally, and is most significant when the distribution is fairly symmetrical, separation of the data into three groups is necessary to avoid wide gaps and extreme skewness. The three groups are (1) those aphids reared in intermittent light and at 24°, (2) those reared in intermittent light at 14°, and (3) those reared in continuous light without distinction of temperature. From two of these have been omitted two percentages (winged aphids in one, intermediates in the other) because they were so high as to create the discontinuity which grouping the data was designed to prevent. These high percentages must have some special reasons with which correlation studies are not fitted to deal. The three coefficients of correlation between percentage of winged and percentage of intermediate-winged are, respectively, $.30 \pm .24$, $-.70 \pm .15$, and $.03 \pm .20$. Two are insignificant, the third strongly negative.

It seems clear, therefore, that whatever is inducing wing development in these more unified segments of the experiments is not particularly conducive to production of intermediates—is, indeed, somewhat opposed to it.

Conceivable Schemes of Stimulation

The general method of explaining intermediates proposed by Goldschmidt (1923 et al.) particularly for intersexes is perhaps applicable to aphids. It involves the assumption that something (a hormone) responsible for the development of structural characters is present in different concentrations at different times, usually in increasing concentrations with increasing age during the critical period of development.

Since the sexes start development under the influence of different groups of genes, and since they typically possess in the end quite different structures, it was natural to assume two hormones, one for each sex. Development of the individual began with one of these hormones in the ascendancy, but somewhere in the course of differentiation it was overtaken by the other hormone, the moment of passing being the "turning point." Structures determined before that point were like those of one sex, characters determined after that point were like those of the other sex.

It would be possible to make the same assumptions regarding intermediates between winged and wingless aphids. These aphids, however, are presumably genetically alike. Moreover, the difference between them lies almost entirely in the possession by the winged type of a group of characters which are wanting in the wingless. It is therefore simpler to represent the differentiating characters as dependent upon the concentration of a single substance in the critical period of development. No serious flaw would be created in the concept of the fundamental nature of the intermediates even if this representation should prove to be in error.

It is assumed for the purpose of this discussion that this one hormone varies in concentration during development in the general manner portrayed by Goldschmidt's curves, which were originally suggested, no doubt, by the course of other biological processes in developing organisms. In proper concentrations it will be regarded as a stimulus to the development of the differential characters of the winged type, though it could as well be considered an inhibitor. The unsegmented egg will be represented as possessing only a minimal quantity of this hormone, though it is conceivable that the egg might have a higher concentration than any later stage. Within these self-imposed limits we may imagine the influence which various situations would have on the frequency and nature of intermediates.

Differentiating Characters.—The winged female aphid differs from the wingless in having wings, three ocelli on the head, wing muscles in the thorax, and extra sensoria on the third segment of the antennæ (4–6 in the wingless, 15–18 in the winged). There are color differences, the third segment of the antennæ being blackish and the thorax brownish in the winged type, but these are subject to considerable variation and are not included in this discussion. It is the four structural characters named which are assumed to develop if a certain concentration of the stimulating hormone is attained early enough.

Sharply Defined Single Thresholds.—The line of demarcation between wingless and winged could be a sharply defined level of concentration of the hormone, such that any deficit, however slight, would leave the differentiating structures wholly absent, while any excess, however small, over this level would cause those structures to be fully developed. The simplest case would be that in which the same concentration of the hormone would stimulate the development of all four structures—wings, ocelli, wing muscles, and sensoria.

The time in development at which these structures have their fate (their presence or absence) decided could likewise be a sharply defined moment, such that if the stimulus were applied in advance of that time the structures would develop, while if the stimulating level of the hormone were attained even only slightly after that time the organs would be entirely lacking in the adult. The simplest case is again that in which the time of determination is the same moment for all four of the structures.

This situation is represented in Fig. 1, *A,* in which time during development is expressed by horizontal distances and concentration of the hormone by vertical distances. The curves indicate the rate of accumulation of the hormone during development. Any curve passing to the left of and above the dot in the middle of the chart would be that of a winged adult; any curve below and to the right, that of a wingless adult.

Intermediate individuals would be practically excluded by the relations here described, since very seldom would a curve of concentration pass exactly through the intersection of stimulation level and time of determination.

Different Times of Determination.—The preceding situation could be modified by having different times of embryonic determination for the four structures, but the same concentration of the hormone as a stimulus for all. This condition is represented in Fig. 1, *B.* The order of determination of the four differentiating features is arbitrarily assumed purely for purposes of illustration. All curves of hormone concentration rising rapidly enough to pass the stimulating level in advance (to the left) of all these times of determination would be those of winged aphids; while all curves reaching that level to the right of (after) all the times of determination would be those of wingless individuals. Curves between these limits would lead to mosaics. The second curve in *B* would be that of an aphid having ocelli, wing muscles and extra antennal sensoria fully developed, but lacking wings entirely. The third curve would pertain to one lacking wings and ocelli altogether, but having wing muscles and extra sensoria fully present. The fourth curve would belong to an aphid having extra antennal sensoria to the full number, but lacking all the other marks of a winged individual.

It is proposed in this paper to use the term mosaic for a patchwork of typical parts, the word intermediate for an individual in which one or more structures are partially developed. "Typical parts" means, in wingless aphids, the absence of any degree of the marks of a winged aphid. A mosaic thus would have one or more of the features of the winged aphid completely developed, but lack the others entirely. One individual could be both mosaic and intermediate, by having certain structures either fully developed or wanting, other structures partially developed.

The conditions described in *B* would lead to a certain number of mosaics, but rarely to intermediates. How many mosaics there were would depend partly on the lengths of the intervals between the times of determination.

Different Levels of Stimulation.—The several features of a winged aphid could be stimulated to develop by different concentrations of the hormone. If there were a single time of determination for all four of these features, the general situation would be as shown in Fig. 1, *C*. Without further explanation it should be clear that the second and third curves are those of mosaics, the second possessing ocelli, wing muscles and extra sensoria, the third having only wing muscles and sensoria. Intermediates would be rare, and the number of mosaics would depend on the extent of the differences among the levels of stimulation.

Range of Stimulation.—It seems likely that a certain concentration of the hormone could not act suddenly to determine a complete structure. More probably a given concentration, if applied early enough, serves to stimulate a slight development of the structure, while a greater concentration would cause a greater development of it. There would thus be a level (concentration) of beginning differentiation, and a level of complete differentiation. If this range of stimulation were identical for all four of the features of the winged aphid, and if there were a sharp time of determination likewise common to all four of them, the conditions would be represented by Fig. 1, *D*. Under these conditions there could be some intermediates, but no mosaics. Any individual whose hormone concentration rose above the level of beginning differentiation before the time of determination, but did not rise as high as the level of complete differentiation until after that time, would be an intermediate. All four of the distinguishing structures would be only partially developed. They might not be developed to equal degrees, since they might respond at different rates to increasing quantities of the hormone. The second curve in *D* would belong to an aphid having wings, ocelli, wing muscles, and antennal sensoria highly, but not completely, developed, the third curve to one in which these structures were only slightly differentiated.

How many intermediates were produced would depend in part on

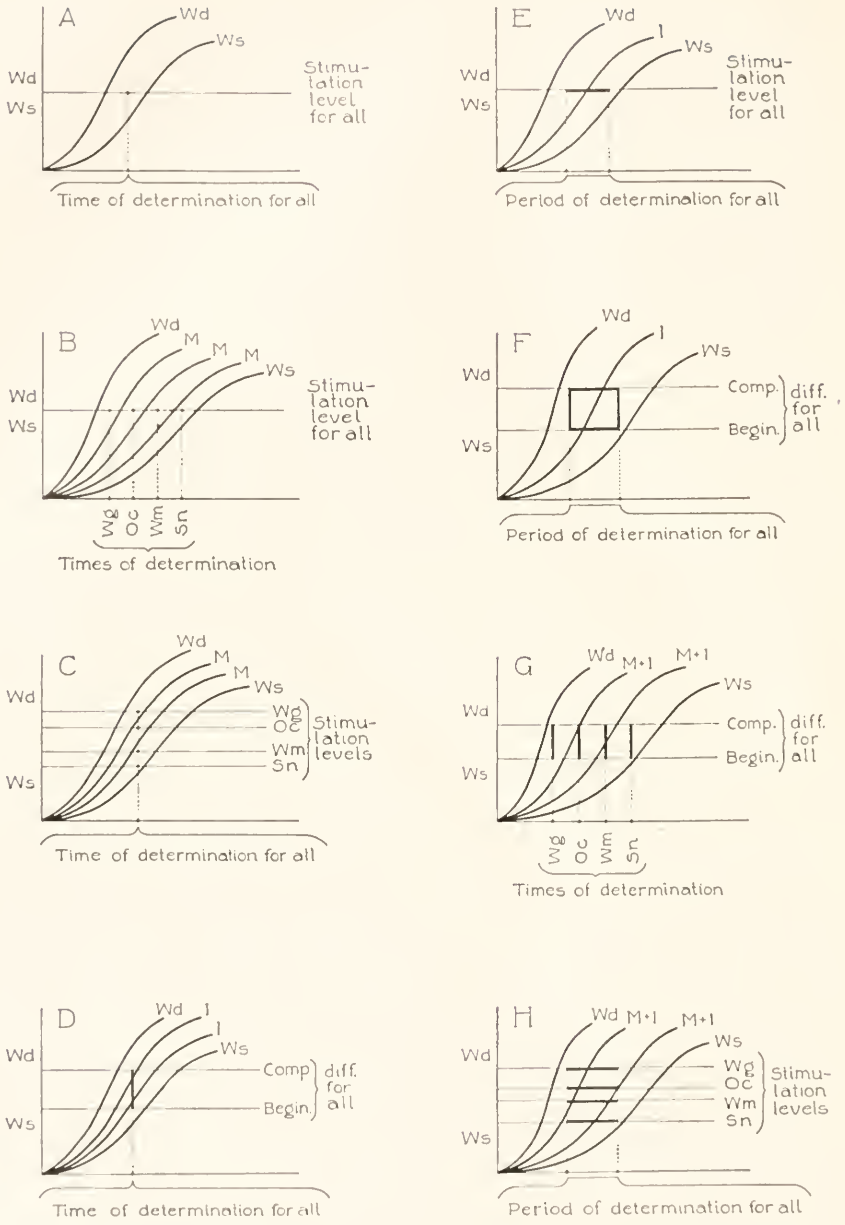


FIG. 1. Curves representing hormone concentration in relation to production of intermediate-winged individuals in aphids, under various assumptions concerning times or periods of determination and levels or ranges of stimulation during embryonic development. *I*, intermediate; *M*, mosaic; *Oc*, ocelli; *Sn*, sensoria; *Wd*, winged; *Wg*, wings; *Wm*, wing muscles; *Ws*, wingless.

the difference between the concentration which would begin and that which would complete the differentiation.

Period of Determination.—It is not necessary that a structure have its fate irrevocably fixed at one moment in development. That determination may be spread over a period of time. If the hormone rises to a stimulating concentration just before that period ends, the structure may be supposed to be slightly developed. If the hormone reaches a stimulating level soon after the beginning of the period of determination, the structure would be highly but not completely developed. In Fig. 1, *E*, it is assumed that there is one period of determination for all structures and one sharp level of stimulation for all of them.

The above situation would lead to the production of some intermediates, but no mosaics. The middle curve of *E* would be that of an aphid having perhaps half-developed wings, ocelli, wing muscles, and extra sensoria. How many intermediates there were would depend on the length of the period of determination.

Range of Stimulation and Period of Determination.—There may conceivably be a range of stimulation levels (as in *D*) and also a period of determination (as in *E*). The simplest such situation is one in which all four differentiating structures would be stimulated by the same range of concentrations of the hormone and be determined during the same period of time. It is represented in Fig. 1, *F*. Any curve of hormone concentration which passed through the rectangle formed by the intersection of the range of stimulation and the period of determination would be that of an intermediate. All the differentiating structures of such an individual would be partially developed, though not necessarily to an equal extent. There would be no mosaics. How many intermediates were produced would depend partly on the size of the rectangle —the extent of the range of stimulation and the length of the period of determination.

Range of Stimulation with Different Times of Determination.—If there be one range of stimulating concentrations of the hormone common to all four of the structures, and sharply defined times of determination which are different for all four, the conditions are those portrayed in Fig. 1, *G*. Any curve cutting one of the heavy vertical lines would belong to an individual intermediate for the structure whose time of determination and range of stimulation that heavy line represents. The same individual would be mosaic for any structures whose heavy line its curve did not cut. Thus, the second curve in *G* would be that of an aphid lacking wings, having almost complete ocelli, and having fully developed wing muscles and sensoria. The third curve would belong to an individual lacking wings and ocelli, but having partially developed wing muscles and fully developed sensoria.

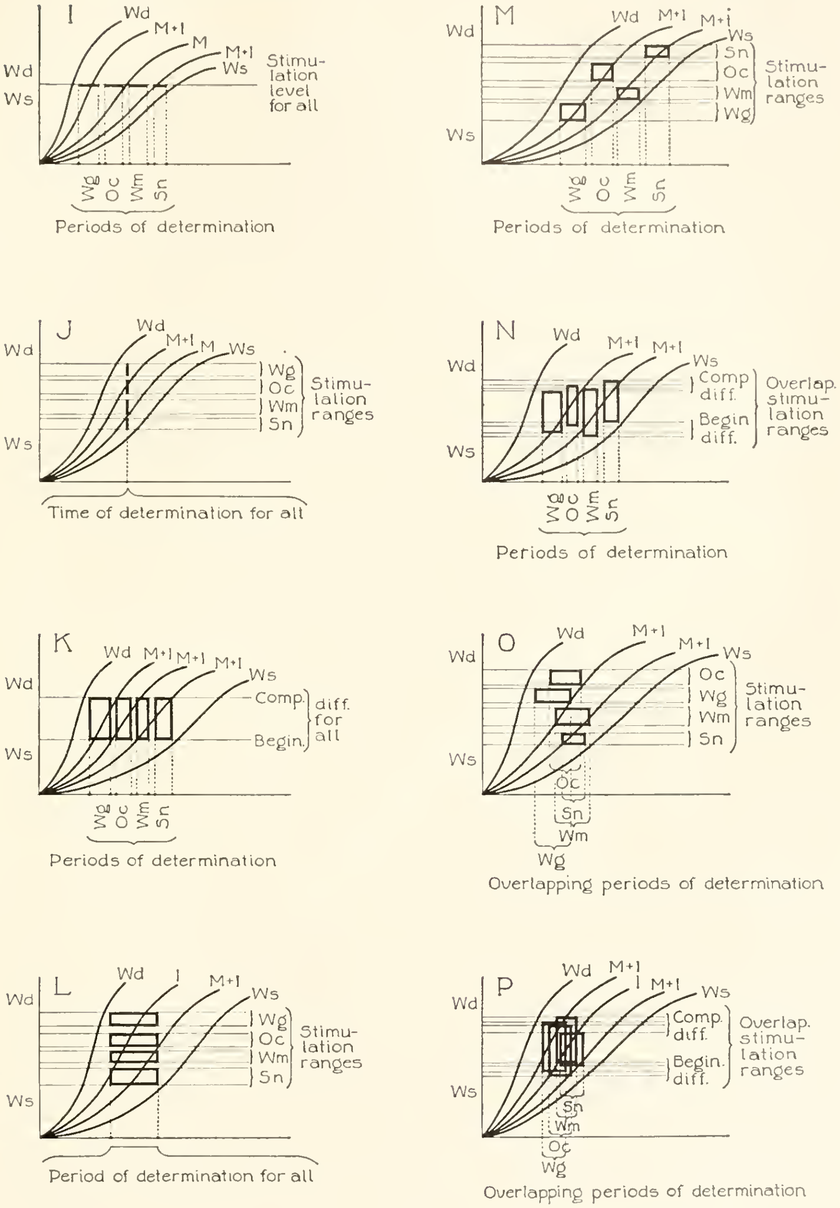
Individuals not typically winged or wingless, produced under the above conditions, might well be both mosaic and intermediate. Some of them would perhaps be mosaic alone, especially if the range of stimulation were narrow and the times of determination were widely spaced. Most of them would be intermediate only, if the range of stimulation were broad and the intervals between times of determination were very short.

Common Period of Determination with Different Levels of Stimulation.—Another possibility is that all structures have the same period of determination but different sharply defined levels of stimulation, as in Fig. 1, *H.* An individual would be intermediate for a given character if its hormone curve cuts the heavy line representing its period of determination and level of stimulation. It would be mosaic for (either possess or lack) any structure whose corresponding heavy line it did not cut.

Intermediates would prevail if the period of determination were long and the stimulation levels close together. Mosaics would be more common if the period of determination were short and the levels of stimulation far apart. Many atypical individuals would doubtless be both mosaic and intermediate.

One Level of Stimulation with Distinct Periods of Determination.—A single sharp level of stimulation for all distinguishing structures may be combined with periods of determination which are different for the different characters. If these periods do not overlap, the situation is as pictured in Fig. 2, *I.* The second curve in *I* is that of an individual with partially developed wings and the other three features of a winged aphid fully formed. The third curve is that of a mosaic having wing muscles and sensoria fully developed but wholly lacking wings and ocelli. Intermediates would prevail among atypical individuals if periods of determination were long and close together; mosaics would lead in numbers if periods of determination were short and separated by wide intervals.

Single Time of Determination with Separate Ranges of Stimulation.—If all the differentiating structures were determined at the same moment, but each had its own range of stimulation which did not overlap any of the others, the situation would be that represented by Fig. 2, *J.* The second curve of this chart, cutting one of the heavy lines representing time of determination and range of stimulation but missing the others, represents a mosaic and intermediate individual. The third curve, missing all the heavy lines, is that of a mosaic only. Intermediates would be favored, among atypical aphids, by wide ranges of stimulation close together; mosaics would be favored by narrow ranges widely spaced.


I
Wd
M+1
M
M+1
Ws
Stimulation level for all
Wd
Ws
Wg
Oc
Wm
Sn
Periods of determination
M
Wd
M+1
M+1
Ws
Sn
Oc
Wm
Wg
Stimulation ranges
Wd
Ws
Wg
Oc
Wm
Sn
Periods of determination
J
Wd
M+1
M
Ws
Wg
Oc
Wm
Sn
Stimulation ranges
Wd
Ws
Time of determination for all
N
Wd
M+1
M+1
Ws
Comp diff.
Begin diff.
Overlap. stimulation ranges
Wd
Ws
Wg
Oc
Wm
Sn
Periods of determination
K
Wd
M+1
M+1
M+1
Ws
Comp.
Begin.
diff. for all
Wd
Ws
Wg
Oc
Wm
Sn
Periods of determination
O
Wd
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M+1
Ws
Oc
Wg
Wm
Sn
Stimulation ranges
Wd
Ws
Oc
Sn
Wm
Wg
Overlapping periods of determination
L
Wd
I
M+1
Ws
Wg
Oc
Wm
Sn
Stimulation ranges
Wd
Ws
Period of determination for all
P
Wd
M+1
I
M+1
Ws
Comp. diff.
Begin. diff.
Overlap. stimulation ranges
Wd
Ws
Sn
Wm
Oc
Wg
Overlapping periods of determination

FIG. 2. Continuation of Fig. 1, representing increasing substitution of ranges for levels of stimulation, and of periods for times of determination, and increasing overlapping in place of distinct ranges and periods.

One Range of Stimulation with Distinct Periods of Determination.—If stimulation occupied a range common to all structures, and each organ had a different period of determination without overlapping, Fig. 2, *K*, would represent the conditions. The three curves in the middle are those of both mosaics and intermediates, since each curve cuts one or more of the heavy rectangles and misses one or more others. If the rectangles were narrow and high and close together laterally (that is, if periods of determination were short and not far apart while the threshold of complete differentiation were far above the threshold of beginning differentiation), most atypical individuals would be intermediate only. If periods of determination were short and widely separated, and the range of stimulation were narrow, mosaics would predominate.

Common Period of Determination with Distinct Ranges of Stimulation.—The situation indicated by this title is portrayed in Fig. 2, *L*. Individuals would be intermediate for those characters whose rectangles their hormone curves traverse, mosaic for those whose rectangles are missed altogether. If the common period of determination were long, and the ranges of stimulation were narrow and close together, most atypical aphids might well be intermediate only. In most other situations of this general kind, combined mosaics and intermediates would be more likely.

Distinct Periods of Determination and Separate Ranges of Stimulation.—In Fig. 2, *M*, is shown the combination of different periods of determination and different ranges of stimulation, without any overlapping of either periods or ranges. Many possibilities for either few or many intermediates, few or many mosaics, are presented by this situation. If the structures having early determination have low stimulation ranges, and those determined late have high stimulation ranges, so that the rectangles are arranged along a line rising obliquely to the right (as is more or less true in *M*), and if the curve of concentration of the hormone follows approximately this same course, the curves would cut most of the determination-stimulation rectangles if they cut any of them, and mostly intermediates would result. An atypical individual might easily be intermediate for all the distinguishing characters. If, however, structures determined early had high ranges of stimulation, and those determined late had low ranges of stimulation, so that in the figure the rectangles of determination and stimulation were arranged along a line sloping downward to the right, cutting the curve of hormone concentration nearly at a right angle, mosaics would prevail if the rectangles were small, and mosaic-intermediates would be most common if the rectangles were large. Many other relations between the period of determination and range of stimulation could exist, such that the rectangles would be distributed irregularly in relation to the hormone curve. Each of these sets of relations would result in a particular frequency of mosaics or intermediates or both.

Distinct Periods of Determination with Overlapping Ranges of Stimulation.—The opportunity for pure mosaics would be reduced by having the ranges of stimulation overlap. This condition along with distinct periods of determination, is shown in Fig. 2, *N*. Unless the overlapping of ranges were very slight, it would be difficult for the hormone curve to pass between rectangles. While curves near the winged or the wingless extreme might miss some of the rectangles, they could scarcely miss all of them and still belong to atypical individuals.

Overlapping Periods of Determination with Distinct Ranges of Stimulation.—Equally difficult, with the preceding situation, for the production of pure mosaics would be the condition in which the periods of determination overlap while the ranges of stimulation are separate. In Fig. 2, *O,* where this situation is represented, there might appear to be a greater chance for one of the atypical curves to miss all the rectangles than in *N,* but that is only because the periods of determination have been assigned a smaller spread than was given to the ranges of stimulation in *N*. Most atypical aphids produced under these conditions would have to be intermediate with respect to some characters, and could easily be intermediate for all their distinguishing marks.

Overlapping of Both Periods of Determination and Ranges of Stimulation.—Still more favorable to intermediates, as compared to mosaics, than any of the preceding arrangements is that in which both the periods of determination and the ranges of stimulation overlap, as in Fig. 2, *P*. If the limits of the several periods and the several ranges did not differ very greatly, there would be few atypical curves that did not traverse several of the determination-stimulation rectangles, and many would traverse all of the rectangles. Intermediates would thus predominate over mosaics, though individuals both intermediate and mosaic could be fairly common.

The above are not all of the conceivable combinations of stimulation and determination. They will suffice, however, to illustrate the possibilities farther than there is any present possibility of forming judgments from them.

Nature of the Production of Atypical Forms

Since the factors which govern morphogenesis are as yet only very imperfectly understood, the possibilities outlined in the preceding section may all be taken into account. What information, if any, concerning them is furnished by the methods of producing the intermediates?

Analysis of the characters of the atypical aphids has only begun, and it is too early to attempt to draw from them the conclusions to which complete study may lead. Already, however, it is clear that most if not all of them are intermediate in one or more respects. To what extent they may also be mosaic is not at present known, but very few of them are pure mosaics.

This fact should mean that the first few situations represented in the curves of Fig. 1 are less likely to exist than are the later ones or those of Fig. 2. Since sharply defined times of determination and levels of hormone stimulation would lead to mosaics, the facts so far ascertained favor the existence of a spread in one or both of these elements. Either there must be a difference between the concentration of the stimulating hormone which will lead to slight development of a structure and the concentration which will complete it, or the amount of development must depend in part on how long the effective stimulation is applied, or both of these statements must be true. Only a more complete knowledge of individual intermediates will indicate how much spread is required.

Until some physiological basis of embryonic determination[2] in the aphids is discovered, either a range of stimulating concentrations or a gradual process of determination may be postulated to explain intermediate individuals. Which is the more likely, or are both probable?

One fact reported in this paper which appears to bear on this question is the large difference in the capacity of the two strains used to produce intermediates. The 1931 line produced less than one-tenth of the number of intermediates produced by the 1923 strain. Also, this capacity to produce intermediates must be subject to large modifications, since the high capacity of the 1923 line (clone A', Shull, 1932) arose suddenly by a " mutation " from a stock (clone A) which was producing apparently no more atypical individuals than the 1931 strain now produces. Which of the possible bases of intermediacy is more likely to exhibit large differences in different strains, or more likely to be subject to considerable change?

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[2] The word " determination " is used in this paper despite its obvious defects. Since different events occurring at different times may be necessary antecedents of a morphogenetic change, a structure could not be said to be actually determined until the last such event had taken place. A morphological feature may be determined, as far as one agent is concerned, later than it is determined in relation to another agent. Were it possible, in the aphid experiments, to designate a single agent in relation to which determination was being considered, a better term that would not be too cumbersome could be devised. But the tables presented in this paper indicate that a complex set of circumstances is involved. Any substitute for the word determination that would be an improvement upon it would be unwieldy. Biologists are not likely to be misled, in this connection, by the briefer term.

Intermediacy could be made more frequent by increasing the length of the period or periods of determination, or by increasing the range of stimulation, so that more curves of concentration of the hormone would pass through them. It could also be made more abundant by bunching the curves of concentration; that is, by creating a set of conditions which would cause larger numbers of individuals to develop their stimulating hormone at approximately the same rate, which would have to be a rate that would make the curves of concentration traverse the lines or rectangles representing periods of determination or ranges of stimulation or both. This concentration of the hormone curves in a narrow band would have to occur in one strain of aphids, but not in another subjected to the same combination of light and temperature, or any other combination yet tested.

The second of these postulates, the clustering of the rates of hormone development, involves an increase in the measure of control exerted by the animals over the process of development. A strain of aphids in which more individuals developed in the same way would be exercising more regulation than would a line in which hormone increase was more nearly random. Now, the 1923 strain, which is the one in which such clustering of rates of hormone development would have to occur, does not regulate wing production nearly as precisely as does the 1931 line. Wing development responds to light conditions much more definitely in the latter than in the former, as a glance at the respective tables shows. The fact that the effect of intermittent light is reversed in the two strains is not at issue; the significant point is that the effect is much more precise in the 1931 strain than in that of 1923. It seems scarcely likely, therefore, that the control over rate of hormone concentration would be greater in the older line than in the newer one.

If this conclusion is justified, there is left the extent of the period of determination or of the range of stimulating concentrations of the hormone, or both, to account for intermediates. Nothing in the experiments here reported appears to provide a choice as between these two possibilities. However, embryonic development is known to consist in some animals, at least in part, of a chain of events in which some or all of the events provide the stimuli leading to later events. To whatever extent this is true, it would be difficult to imagine any great extension of the periods of determination of structures. Were many of these to be extended, and were each to wait upon the culmination of some preceding event, embryonic development would be correspondingly prolonged. Nothing is known that would render it probable that the embryos which become intermediate or mosaic adults take longer to develop, though the numerous ovarioles would make it possible for that to happen and for the young aphids to be borne out of their regular turns based on the time of starting cleavage. On the whole, it would seem unlikely that embryonic development is retarded greatly by the factors leading to intermediacy.

The concentration of the hormone, on the contrary, is subject to no such known limitation. So far as anyone knows, the level of stimulation of completed differentiation could be farther above the level of beginning differentiation in one line of aphids than in another. So far as known there is no chain of events involved in the concentration of the hormone, such that one structure cannot begin to be stimulated to develop before the stimulation of another is completed.

It is suggested as more probable, therefore, that intermediacy in these aphids depends upon a gap between the thresholds of stimulation of beginning and of complete differentiation, rather than upon periods of determination or clustering of the rates of hormone development.

How this conclusion is related to the differences in the number of intermediate individuals induced by environmental conditions is difficult to ascertain. If the general concept that hormone concentration and range of stimulation levels combine to determine the winged type is correct, higher proportions of intermediates could be produced at the expense of winged aphids by lowering the hormone curves (reducing the rate of accumulation of the hormone) or at the expense of wingless aphids by elevating the hormone curves—all on the assumption, made to simplify the discussion, that the hormone is a stimulant rather than an inhibitor of the development of the characters of the winged females. The more striking changes in the number of intermediates produced by light and temperature in the 1923 strain were in general of a sign opposite to that of the change in the number of typical winged females produced by the same conditions. The suggestion contained in this comparison is that intermediates were being produced at the expense of winged adults. The reduction of the winged ones was in general, however, several times as great as the increase of the intermediates. In other words, many winged aphids were being converted into wingless ones as well as some into intermediates. This fact is not opposed to the supposition that hormone accumulation was being retarded; but since there were many wingless in most environmental conditions, a mere lowering of hormone curves would not necessarily leave any more of them in the intermediate zone. It would seem necessary to suppose that, in the lowering of the hormone concentrations, there was either an accumulation of the curves in the intermediate zone, or a widening of the ranges of stimulation so that more curves would intersect them.

One reason for not assuming an accumulation of hormone curves in the intermediate regions has already been pointed out.

It is worthy of note that in the 1931 strain, the only set of conditions which induced many intermediates served to increase, rather than diminish, the number of winged individuals (from wingless parents) or had practically no effect on the number of winged ones (from winged parents). If the explanations discussed above were to have general validity, it would be necessary to suppose that the intermediates from wingless parents were being produced at the expense of wingless ones, which might be due in part to a general lifting of the hormone curves. This contrast in the behavior of the two strains recalls their different responses to intermittent light in the production of wings; intermittent light produces fewer winged aphids in the 1923 strain, but more winged ones in the 1931 strain. There may be some common basis for these two differences. What reversal would be required in the hormone scheme to account for these contrasts it is impossible to say with any high degree of probability.

Whether intermediates are in any way dependent on the *direction* of some physiological change (that is, winged to wingless, or wingless to winged) can hardly be judged from the experimental methods of producing them. Analysis of the characters of the intermediates will be needed for an answer to this question.

SUMMARY

Two strains of aphids were subjected to various combinations of temperature and light. In one strain (collected 1923) distinctly more intermediate-winged females were produced in intermittent than in continuous light, in every combination of other conditions. Among those reared in intermittent light, more intermediates were produced at a temperature of 14° than at 24°, in every combination of other conditions. Those reared in continuous light, however, produced usually fewer intermediates at 14° than at 24°, and the differences were small. In general, winged parents produced more intermediate offspring than did wingless parents, but there was much irregularity of this relation. Greatest production of intermediates and greatest production of typical winged females did not occur at the same time; there was, indeed, a tendency for the two frequencies to be opposed to one another. Other relations in this strain were rather indefinite. In the second strain (collected 1931) more intermediates were usually produced in intermittent than in continuous light, but the differences were small. More intermediate offspring were produced by parents drawn from a stock kept at

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room temperature than by parents taken from stocks kept continuously at 14° or 24°, or from a stock regularly alternated daily between 14° and 24°, though again most of the differences were small. The greatest (and the only large) number of intermediates in the 1931 strain were produced by taking aphids from a stock kept at 24°, changing them for one generation to room temperature, then putting this latter generation, when adult, at 14°. In general, in this strain the greatest frequency of intermediates occurred when the frequency of typical winged aphids was medium. Other relations in the 1931 strain were not striking.

If it be assumed that production of the characteristics of winged aphids be dependent on suitable concentrations of a hormone which increases in amount during development (other assumptions could be made), intermediacy or mosaicism might result from various combinations of times or periods of determination, or of levels or ranges of concentration of the stimulating hormone, or both. Ranges of stimulating concentrations are less subject to known limits than are periods of determination, and are held to be the more likely, inasmuch as strains of aphids differed greatly in their tendency to produce intermediates. Intermediacy, as contrasted with mosaicism, is favored by extended periods of determination and ranges of stimulation, especially if there is overlapping of these periods and ranges for the several structures.

In part, the intermediates may replace winged aphids in the 1923 strain, and wingless ones in the 1931 strains.

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THE LOPING OF LAND-SNAILS

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A little more than three decades ago Carlson (1905) published a very circumstantial account of the locomotion of a Californian land-snail, *Helix dupetithouarsi*, which may be described as a lope. The animal by appropriate muscular contractions lifts its head well above the substrate, projects it forward much in advance of its former position, and brings it down again on the surface over which it is progressing. Thus the anterior third of the snail forms an arch of which the head is one base and the mid-region of the foot the other, the part between being lifted well off the ground and representing the region of principal muscular action. This arch makes up one step in the snail's forward locomotion and passes over its foot and body in an antero-posterior direction to disappear at the tail. According to Carlson as many as three such arches may be seen progressing as waves over the snail at once. This lope results in unusually rapid progression and is employed by the animal in place of its ordinary slow creeping as a means, for instance, of escaping from an enemy.

In 1911 I called attention to this remarkable form of progression as described by Carlson and expressed the opinion that in the lope the large waves were probably combined with the waves for slow locomotion, a point not touched on by Carlson. I also pointed out that the loping waves took what has been designated as a *retrograde* course, that is, they ran from anterior to posterior, whereas in helices generally the ordinary locomotor waves are *direct*, from posterior to anterior. To clear up the question of the possibility in snails of a double set of waves, one opposite in direction to the other, and to elucidate further matters connected with so exceptional a scheme of progression, called for an examination of living specimens of *Helix dupetithouarsi* which at that time was impossible for me.

During a period of work at the Kerckhoff Laboratories of the California Institute of Technology in the spring of 1936, I noticed in the early morning on the brick walks of the campus interrupted snail trails of the kind figured by Carlson (1905) for this loping species. I immediately made a search for the snails and soon found living specimens in the act of making broken trails. The loping waves on these snails ran from anterior to posterior as described by Carlson. The maximum number of them seen on the foot at once was three, the foot itself being about 4.5 cm. long. When the snail was in full action the arches of the body were so high that light from the early morning sun could be seen shining under them. These arches at times tended to become obliterated toward the hind end of the foot and in some instances they ceased after having passed over only the anterior half of the pedal surface, but as a rule they continued with full vigor all the way to the hind end. As a result of these differences the trails made by the snail varied from a succession of isolated spots through a series of connected bead-like spots to a continuous trail of uniform width. Such variations were to be noted in different parts of the single trail of a given individual, showing that from time to time the snail changed its type of locomotion.

A number of these snails were brought into the laboratory for further and closer study. Here they were made to creep upon large plates of glass where they could be induced to exhibit the same loping locomotion that they had shown on the damp brick paths. A single loping wave was found to pass over the foot from anterior to posterior, a distance of about 4.5 cm., in from eight to ten seconds. The snail while loping covered about ten centimeters in four minutes.

With the snail creeping on a glass plate it was possible to observe the foot from below and to record its action. In ordinary creeping the foot was covered by a succession of small transverse waves, about six in all, which passed from posterior to anterior and coursed over the length of the foot in from five to six seconds each. These both in direction and in general character agreed with the type of locomotor wave long since described for the majority of helices. When the snail began to lope, the large waves were easily followed from below the glass plate. Though the direction which they took over the foot was the opposite of that taken by the smaller waves, the two systems ran simultaneously and without interference. Thus the underside of the foot showed at once a system of small pedal waves running from behind forward, direct waves, and another of large loping waves running from anterior to posterior, retrograde waves. As a result of this double system the animal moved forward rapidly. It is hardly necessary to remark that the direction in which any such set of waves runs is independent of the direction in which the part of the foot that is concerned with the locomotor step moves. In both sets of waves the portion of the foot that is helpful in locomotion moves anteriorly; in the loping waves this locomotor action begins at the anterior end of the snail's body and foot and proceeds posteriorly and in the ordinary creeping waves it begins in the posterior part of the foot and proceeds anteriorly. The musculature involved in the two systems of locomotion must, of course, be distinct; that for loping must include the body musculature directly dorsal and lateral to the foot and that for ordinary creeping the musculature immediately in the foot itself and next its creeping sole. Of the scores of instances of loping that I have observed from the underside of the glass plate, I have never seen the two systems of waves in any other relation than that just described. Further, I have never seen a snail in motion that has not exhibited the system of small waves. These are an invariable accompaniment of locomotion. When loping is undertaken, it is always superimposed on the smaller wave-system though without disturbance. Loping never occurs except after the snail has begun ordinary creeping. Thus the question left open by Carlson as to the relation of these two types of locomotion receives its answer.

I preserved the shells of the snails with which I worked in Pasadena and on my return to Cambridge, Massachusetts, I referred them to Mr. W. J. Clench of the Museum of Comparative Zoology for identification. Much to my surprise he informed me that they were the shells of *Helix aspersa,* a common European snail which was known to have been introduced into California some forty years ago and specifically into the region about Los Angeles. On looking up its habits I found in Taylor's monograph of British land and freshwater mollusks (1914) a brief statement with figures attributed to L. E. Adams showing that the English representatives of this species were known to lope.

This discovery led me to suspect that possibly Carlson in his original investigation had really worked on *Helix aspersa* and not on *Helix dupetithouarsi.* However, through the kindness of Mr. Clench I received a number of living *Helix* (now *Epiphragmorpha*) *dupetithouarsi* from Cypress Point, Monterey County, California, and I had the opportunity of testing these snails as I had done with *Helix aspersa.* They loped as *H. aspersa* did and I could confirm all the statement made by Carlson for *H. dupetithouarsi.* Moreover, this species, like *H. aspersa,* always showed the ordinary locomotor waves when it crept and never loped except when these waves were present. In both species, then, loping appears to be a form of locomotion literally superimposed on ordinary creeping and a means of accelerating progression beyond that attainable by ordinary methods.

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THE PHYSIOLOGY OF DIGESTION IN PLANKTON CRUSTACEA

II. Further Studies on the Digestive Enzymes of (A) Daphnia and Polyphemus; (B) Diaptomus and Calanus

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Introduction

It has been concluded by numerous workers previously quoted by the author (1935), in addition to Gellis and Clarke (1935) and Dehn (1930), that plankton Crustacea feed on particulate food and derive no nourishment from dissolved organic matter, or at least absorb so little of it that dissolved food stuffs account for only a small part of their metabolic needs. This circumstance makes it necessary that these organisms possess an enzyme system adequate for complete digestion of particulate food. Our first studies established the presence of a proteinase, amylase and lipase in *Daphnia*. This paper extends these studies to the peptidases of *Daphnia* and to the proteinases, amylases and lipases of *Polyphemus*, a gymnomerous cladoceran, and to the copepods *Diaptomus* and *Calanus*. Certain experiments to study the behavior of *Daphnia* proteinase at low temperature are also included.

Preparation of Extracts

Daphnia pulex were netted from Lake Monona, Wisconsin, in May 1935 when a pure culture was obtainable in large quantities. A pure culture of *Polyphemus* sp. was obtained from Crystal Lake, Wisconsin, in August 1934. *Diaptomus* sp. was found fairly abundant in a bog lake near Trout Lake, Wisconsin, in July, 1935. *Calanus finmarchicus* was collected from Buzzards Bay, Massachusetts, through the facilities of the Woods Hole Oceanographic Institution.

The glycerol extracts were made from the acetone-dried and petroleum ether-extracted whole organisms after grinding. The water extracts of *Daphnia* were prepared from the fresh, ground organisms and after filtration preserved with toluol.

Peptidases of Daphnia

Our criterion of the presence of dipeptidase was the splitting of leucylglycine, as pointed out by Waldschmidt-Leitz (1931). Some contention exists as to what was called aminopolypeptidase (Johnson et al, 1936), but for convenience this enzyme was considered present when the substrate leucyldiglycine was split, and carboxypolypeptidase was considered present when the substrate chloracetyltyrosine was attacked.

Titrations were made by the Linderstrøm-Lang (1927) method in 90 per cent acetone, using N/10 alcoholic HCl and naphthyl red as indicator.

No attempt was made to purify or separate the peptidases. The crude extract was used in all cases.

Table I

Hydrolysis of Peptides by Crude Extract

Enzyme	Substrate	pH	Hydrolysis* of one linkage in cc. of HCl		
			3 hrs.	12 hrs.	19 hrs.
Dipeptidase	*dl*-leucylglycine	7.2	0.08	0.28	0.54
Aminopolypeptidase	*dl*-leucyldiglycine	7.2	0.15	0.42	0.55
Carboxypolypeptidase	chloracetyl-l-tyrosine	7.2	0.03	0.11	——

* Hydrolysis of one linkage of one component is indicated in the two *dl* mixtures.

The enzyme-substrate mixture for dipeptidase determination consisted of 5 cc. of N/10 leucylglycine in phosphate buffer pH 7.2, and 2 cc. of 1 per cent *Daphnia* water extract. This was incubated at 34° C. and at intervals 2 cc. of the digest was removed and titrated. An HCl titration figure of 0.71 cc. was equivalent to 100 per cent hydrolysis of one linkage of one component. Table I shows the progress of hydrolysis in cc. of HCl.

For aminopolypeptidase 4 cc. of N/10 leucyldiglycine, and 2 cc. of 1 per cent water extract and 1 cc. of water were incubated at 34° C. Two cc. aliquots were withdrawn at intervals and titrated. An HCl titration figure of 0.57 cc. was equivalent to 100 per cent hydrolysis of one linkage of one component. Hydrolysis is indicated in Table I.

A limited supply of substrate for determination of carboxypolypeptidase prevented more than one analysis. Four cc. of M/30 chloracetyltyrosine, 2 cc. of water extract and 1 cc. of water were incubated at 34° C. Two-cc. samples were titrated at intervals.

It is perhaps true that the activity of these water extracts of *Daphnia* was considerably less than the activity of the enzymes *in vivo*. Several experiments on tissues and secretions of the hog and dog indicated that the activity of the enzyme preparation was in the order: (1) secretion, (2) extract of fresh tissue, (3) extract of dry tissue.

Effect of Temperature on Daphnia *Proteinase*

Daphnia is active in lake waters of low temperature. In fact they are known to spend the major part of the day in the hypolimnion of stratified lakes, where the temperature is as low as 4° to 8° C. The question arises as to whether their enzymes are particularly adapted to activity at low temperatures. In order to test this, extracts of a warm-blooded animal (hog) were tested at 20° and at 8° C. and the depression of activity compared with a similar depression of activity of *Daphnia* proteinase.

TABLE II

Activity of Daphnia and hog proteinase at 20° and 8° C. The results are expressed in milligrams of trichloracetic acid soluble N in 20 cc. of filtrate.

Digestion time	Temperature			
	20° C.		8° C.	
	Daphnia	Hog	Daphnia	Hog
hours				
1	0.28	6.41	0.00	3.23
2	0.81	10.78	0.29	5.28
5	2.56	16.78	0.93	8.90
13	4.18	—	1.78	—
24	6.01	—	2.22	—

One gram of dried, defatted hog pancreas was mixed with 100 cc. of 50 per cent glycerol. After standing at room temperature for 24 hours the mixture was filtered through silk bolting cloth. Ten cc. of this filtrate were added to 125 cc. suspension of casein in water (10 gm./100 cc. H_2O) and kept at 20° C. Twenty-five-cc. samples were removed at intervals and precipitated with 35 cc. of 10 per cent trichloracetic acid. The suspended protein was filtered off and the total nitrogen (Kjeldahl) was determined in duplicate on 20 cc. of the filtrate.

A similar digest was set up at 8° C. and the same procedure carried out. The bath consisted of running Lake Mendota water whose temperature was 8° C. at the time of the experiment.

Glycerol extracts of dried, defatted, ground *Daphnia* were prepared by extracting 1.0 gm. of *Daphnia* with 100 cc. of 50 per cent glycerol. This extract was treated in the same manner as described above for hog pancreas.

After 2 hours digestion (Table II), the hog pancreas produced 48 per cent more nitrogen at 20° than it did at 8° C. *Daphnia* proteinase produced 36 per cent more nitrogen at 20° than at 8° C. In 5 hours the respective percentages were 53 and 36. From these data it appears that *Daphnia* proteinase behaves, in general, like that of the hog and follows the van't Hoff law, so that it possesses no super-activity at a low temperature.

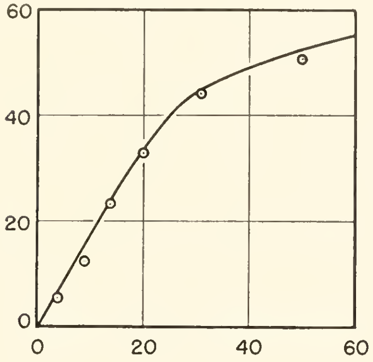

FIG. 1

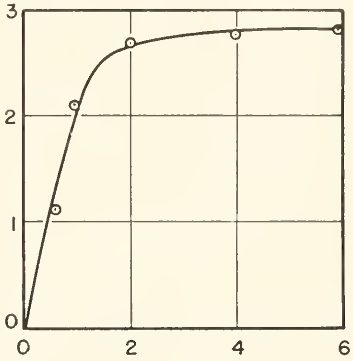

FIG. 2

FIG. 1. Hydrolysis of gelatin at pH 7.2 by a proteinase of *Polyphemus*. The ordinate represents decreasing viscosity ($\triangle V$); the abscissa is the time in minutes.

FIG. 2. Hydrolysis of starch by an amylase of *Polyphemus*. The ordinate is the number of cc. of N/40 $Na_2S_2O_3$, or the amount of N/40 reducing groups; the abscissa is the time in hours of incubation at 37° C.

SOME ENZYMES OF POLYPHEMUS

Proteinase.—A glycerol extract was made from comminuted, dried and defatted *Polyphemus*. One part was diluted to 0.025 per cent for use in proteinase determination and another to 1 per cent for amylase and lipase determination. To 0.5 cc. of 0.025 per cent glycerol extract was added 5 cc. of 1.5 per cent Sargent's gelatin; the mixture was incubated at 34° C. and the viscosity periodically measured in an Ostwald viscosimeter by the method of Northrop (1922). The proteinase was designated as a tryptic enzyme, for it was highly active at pH 7.2, almost inactive at pH 4.35 and completely inactive at pH 3.0. The curve in Fig. 1 indicates the nature of the enzyme activity on gelatin at pH 7.2.

Amylase.—*Polyphemus* extract (1 per cent) was also capable of hydrolyzing starch. Twenty-five cc. of 3 per cent starch at pH 7.2, 25 cc. of water and 5 cc. of 1 per cent glycerol extract were incubated at 37° C. At intervals 10-cc. samples were withdrawn and the reducing groups determined iodometrically according to Baker and Hulton (1920). The results are shown in Fig. 2.

Lipase.—Four per cent tributyrin was attacked by an esterase of the extract. Fifty cc. of 4 per cent tributyrin emulsified with sodium glycocholate and 5 cc. of 1 per cent glycerol extract were placed at 37° C. Ten-cc. aliquots were withdrawn and titrated with N/20 NaOH. The titration figures can be read from Fig. 3.

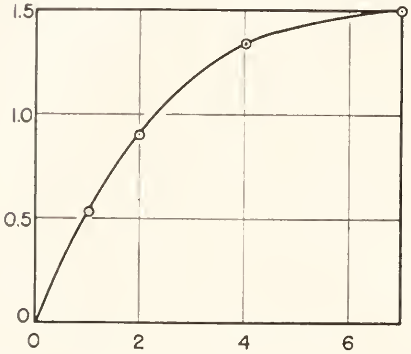

FIG. 3

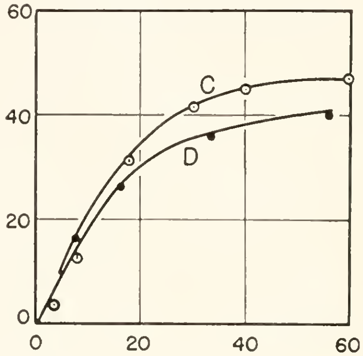

FIG. 4

FIG. 3. Hydrolysis of tributyrin by lipase of *Polyphemus*. The ordinate represents the number of cc. of N/20 NaOH; the abscissa is the time in hours.

FIG. 4. Hydrolysis of gelatin at pH 7.48 by proteinases of *Diaptomus* (*D*) and *Calanus finmarchicus* (*C*). The ordinate represents the decreasing viscosity of the gelatin ($\triangle V$); the abscissa is the time in minutes.

SOME DIGESTIVE ENZYMES OF DIAPTOMUS AND CALANUS

The purpose of this study was to analyse extracts of a marine and a fresh water copepod for enzymes which attack proteins, carbohydrates and fats. An attempt was made with *Calanus* to corroborate the results of Bond (1934) by the use of different extraction and assay methods.

Clarke (1934) has adequately discussed the problem of marine copepod nutrition and has (1935) presented good evidence that their food, as in *Daphnia*, is of a particulate nature. The work of Bond represents a study of some of the enzymes of the marine copepod *Calanus finmarchicus*. Alcoholic extracts that he made were able to hydrolyze gelatin, starch and ethylbutyrate.

Materials

The marine copepod *Calanus finmarchicus* and the fresh water copepod *Diaptomus* sp. were collected at the places mentioned in an earlier division of this paper.

Methods of extraction were identical with those described above for hog and *Daphnia.*

Examination of the Extract

Proteinase.—A proteinase that hydrolyzed gelatin was demonstrated in the extract of *Calanus* and *Diaptomus* by the viscosity method. On the basis of the *Daphnia* proteinase unit (1 unit: 20△V/20, Hasler, 1935), 0.5 cc. of 1 per cent glycerol extract of dried, defatted *Diaptomus* contained 1.5 units. A similar amount of *Calanus* extract had a content of 1.7 units. One per cent extracts of *Daphnia* had 1.5 units. The amounts of enzyme in these extracts appear to be of the same magnitude. The curves in Fig. 4 show that digestion took place within the first few minutes of enzyme activity.

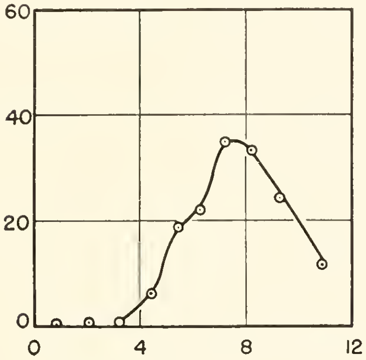

FIG. 5

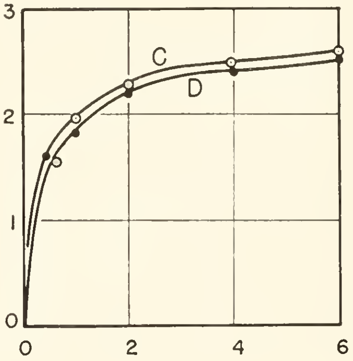

FIG. 6

FIG. 5. The pH activity curve of *Calanus* proteinase on gelatin. The ordinate represents decreasing viscosity of the gelatin (△V); the abscissa represents pH.

FIG. 6. Hydrolysis of starch by amylases of *Diaptomus* (*D*) and *Calanus* (*C*). The ordinate represents the number of cc. of N/40 $Na_2S_2O_3$, or the amount of the N/40 reducing groups; the abscissa is the time in hours of incubation at 37° C.

Sufficient extract was available from *Calanus* to construct a pH activity curve. Figure 5 shows the enzyme to be of definite tryptic type with maximum activity between pH 7–8. Inactivity resulted when the substrate pH was reduced to pH 3.5. Medium activity was evident between pH 4–6 which indicated the presence of a katheptic or autolytic enzyme of the tissues. The alcoholic extracts of *Calanus* made by Bond (1934) were most active at pH 8.0–8.49. The secondary optimum of his extracts at pH 3.6–4.0 were unconfirmed in this work. The secondary optimum resulting from the autolytic enzymes was pH 5.6. The discrepancy of pH optima may be due to variations in extracting technique, for Mansour-Bek (1932) found that crude proteinase extract of *Maja squinado* had a pH optimum on gelatin of 6.0; the optimum for purified enzyme was pH 8.1. The pH of the buffered substrates used in this experiment was determined with the aid of the glass electrode.

Amylase.—Both *Diaptomus* and *Calanus* extracts contained active amylase which hydrolyzed starch. The iodometric titration previously described was used as a criterion of starch hydrolysis. The amounts of N/40 $Na_2S_2O_3$ present in 10 cc. of digest can be read from Fig. 6. These figures are equivalent to the amount of N/40 reducing sugars liberated by hydrolysis. In both instances 5 cc. of 1 per cent glycerol extract were added to 25 cc. of 3 per cent starch at pH 7.2 and 25 cc. of water and incubated at 37° C. At intervals 10-cc. samples were withdrawn and the reducing groups titrated.

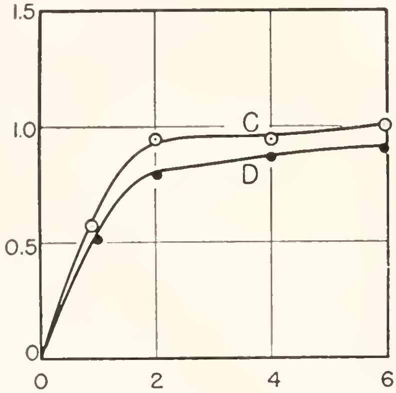

FIG. 7. Hydrolysis of tributyrin by lipases of *Diaptomus* (*D*) and *Calanus* (*C*). The ordinate indicates the number of cc. of N/20 NaOH; the abscissa is the time in hours.

Lipase.—Tributyrin was attacked by an esterase present in both *Diaptomus* and *Calanus* extracts. Fifty cc. of 4 per cent tributyrin were emulsified with sodium glycocholate to which were added 5 cc. of 1 per cent extract of *Diaptomus;* extracts of *Calanus* were examined by the same procedure. At intervals 10-cc. aliquots were withdrawn and titrated with N/20 NaOH. The results are shown in Fig. 7.

Experiments in all instances were duplicated and simultaneously run with adequate controls.

Discussion

Biochemical analyses presented in Table I, and by the author in a previous paper (1935), demonstrate that *Daphnia* possesses a system of proteolytic enzymes which can attack a protein (in this case gelatin and casein) and cleave it to amino acids. This bears out the contention that plankton crustaceans can utilize particulate organic matter.

The experiments on temperature effect on enzyme activity show that the *Daphnia* and hog enzymes follow, in general, the van't Hoff rule that a reaction approximately doubles for every 10° C. increase in temperature. It suffices to conclude from these data, that a low temperature does reduce the entire metabolism of the organism. More data should be collected on this subject, for Wiersma (1928) held that *Astacus* amylase was strongly active at 0° C., while Yonge (1926) found that enzymes of *Ostrea* were inactive at this temperature.

Another cladoceran (*Polyphemus*) and a copepod (*Diaptomus*) have been added to the list of plankton Crustacea which can be definitely said to possess functional digestive enzymes capable of attacking the three important food stuffs. It is quite clear from the review of Krüger (1933) and Yonge (1931) that these entomostracans together with *Daphnia* do not differ from the enzyme types of the larger Crustacea such as *Astacus* and *Maja.*

Although this work concludes the long series of experiments designed to solve the problem of nutrition in plankton Crustacea as challenged by Pütter, there remains an untouched field in enzyme chemistry of Crustacea, namely the kinetics and purification of these enzymes. A study of the kinetics of purified enzymes has been made by Mansour-Bek (1932) on the decapod, *Maja squinado.* She brought to light some interesting similarities between vertebrate and invertebrate enzymes, e.g. purified proteinase was activated by mammalian enterokinase. A continuation of her stimulating work would be an excellent contribution. Difficulties may be expected, however, in working with entomostracans, for the present purification methods require large amounts of extract to carry out proper elution.

Appreciation is expressed to Professor H. C. Bradley for furnishing laboratory facilities and for gladly proffering biochemical advice; also to Professor C. Juday for biological suggestions and personal interest in the problem.

SUMMARY

1. Three peptidases (dipeptidase, aminopolypeptidase and carboxypolypeptidase) were found in water extracts of *Daphnia*. The presence of these and a proteinase (author, 1935) demonstrate that *Daphnia* can completely utilize a protein.

2. Proteinases of *Daphnia* were no more active than those of the hog pancreas at a temperature of 8° C. Both enzyme systems follow, in general, the van't Hoff rule.

3. Proteinases, amylases and lipases were found in glycerol extracts of *Polyphemus* and *Diaptomus*. All proteinases were of the tryptic type with an optimum activity at pH 7–8. They simulate *Daphnia* enzymes and show that plankton Crustacea have complete enzyme mechanisms for digestion of particulate food.

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SYMMETRY AND REGULATION IN MNEMIOPSIS LEIDYI, AGASSIZ[1]

B. R. COONFIELD

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The ctenophores, a group in which *Mnemiopsis* is included, are usually referred to as biradially symmetrical animals. By some they are believed to have primarily a radial symmetry. There is of course the idea of bilateral symmetry expressed in the term biradial. As one sees this animal swimming freely in water it does seem to possess a radial symmetry. This impression is received because its organs are distributed about a central, longitudinal axis. But a close examination of this animal reveals that most of its organs are paired and are located on two opposite sides of the central, single organ, the stomodeum (Figs. 1 and 11). A method other than and including the study of the anatomy of this animal has been used by me in getting information about its symmetry. This information was obtained during a series of experiments which had been planned to show other characters of this animal. The one feature which has been demonstrated repeatedly during experimentation on *Mnemiopsis* is the power to regulate itself. This power to regulate its body following injury either in nature or caused by experiments seems to have some bearing on symmetry. I propose, therefore, to show in this report that *Mnemiopsis* possesses a particular type of symmetry, bilateral symmetry without dorso-ventrality, as well as the power to regulate itself, and that in this animal symmetry and regulation are associated with each other.

EXPERIMENTS

The operations were performed on the specimens and each was kept in a separate finger bowl which was partly immersed in running water. Observations, drawings, and photographs were made at frequent intervals. Only a very few of the experimental animals failed to survive. The changes observed during the reorganization following the operations were recorded in detail. In a great many instances these changes were observed quite easily with only the aid of glasses of low magnification. The drawings were made with the aid of a camera lucida while the photographs were taken with a Leica camera with an extension tube outfit.

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[1] Contribution No. 19 from the Department of Biology, Brooklyn College.

Although the experiments listed and described in this report seem to be quite varied they can be grouped under two headings; experimental cuttings, and experimental graftings.

Experimental Cuttings

The animals were cut across at four levels of the body and only the oral pieces were retained for observation. By this cutting at different levels some of the oral pieces lost only the very apical tip of the animal while others had removed from them the part of the body apical to the bases of the auricles. These experiments were done in accordance with an objective somewhat different from the one included in this report. The details of these experiments will be reported later. However, as the pieces of the animal were passing through the phases of regeneration several results were pertinent to the problem considered herein.

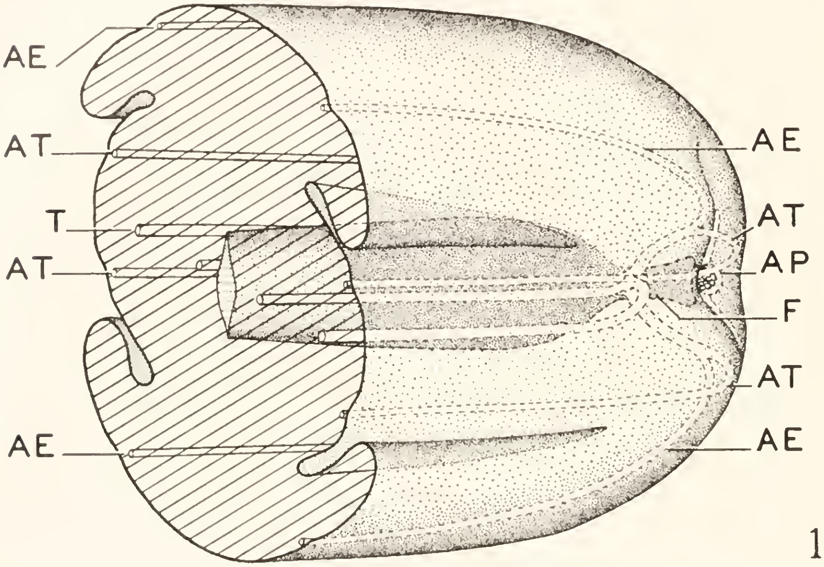
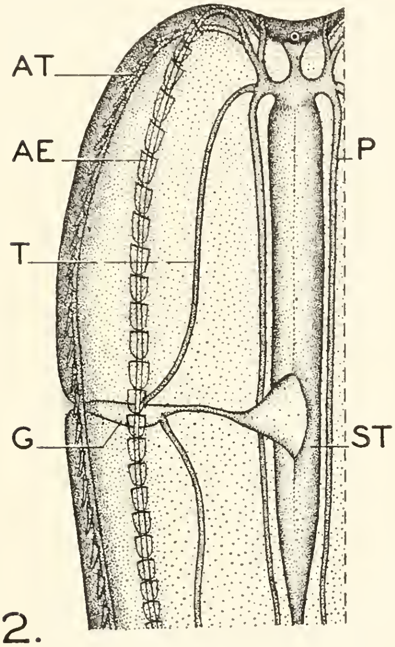


Fig. 1. This is a diagram representing the shape and symmetry of the body of *Mnemiopsis*. The plates and some of the smaller canals are not shown.

AE, adesophageal row; *AP*, apical organ; *AT*, adtentacular row; *F*, infundibulum; *T*, tentacular canal. The stomodeum is shown in the center of the drawing with the paragastic canals lying parallel to and near it.

The oral pieces usually regenerated the lost portions of the body perfectly in all details. By this regeneration the regular connections of canals and rows were soon established and the single, apical organ was formed (Figs. 9, 15 and 17). During regeneration the two adjacent adtentacular rows and a single adesophageal row on either side of these connected to each other. As this was taking place the tip of the stomodeum formed a single bulb (Fig. 18), which immediately divided to form two bulbs (Fig. 4), and each of these became connected with a set of



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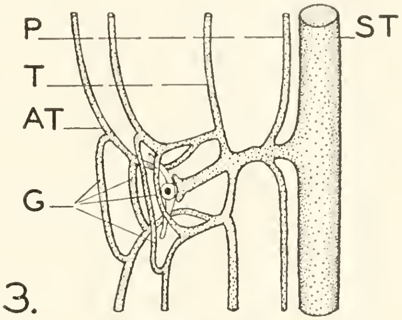

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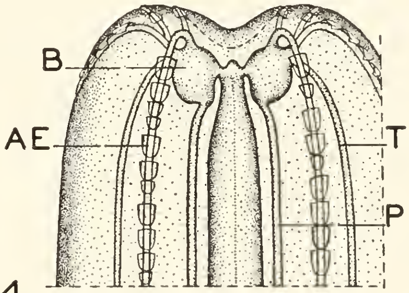

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FIG. 2. An adesophageal view of *Mnemiopsis* showing a stomodeal graft after the second day.

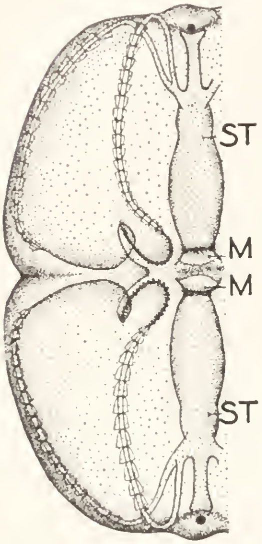
FIG. 3. This drawing shows an apical organ grafted to the adtentacular region and about mid-way between the ends of the body. The drawing was made on the fourth day after the operation.

FIG. 4. This shows the usual features at the apical end of the body during regeneration following the removal of an apical part of the animal. The two bulbs can be seen lying at the upper end of the stomodeum. This drawing was made on the fourth day.

AE, adesophageal row; *AT*, adtentacular row; *B*, stomodeal bulb; *G*, graft; *P*, paragastric canal; *ST*, stomodeum; *T*, tentacular canal.

four canals of the plate rows. The two bulbs became smaller until they assumed the size of a tube or canal and became the radial canals. At about this time also a single, apical organ formed at the tip of the stomodeum between the two bulbs. The apical plate of this organ was regularly laid down on a line between each of the two adjacent pairs of adesophageal rows (Fig. 9).

Certain irregularities were observed on the regenerating oral pieces following the cutting at each of the four levels. These consisted of a failure of the eight rows to connect as a unit resulting in the formation of two apical organs (Figs. 7 and 19), a failure of the usual row connections with or without an apical organ (Figs. 10, 14 and 16), and a splitting of the tip of the stomodeum resulting in a separation of the opposing pairs of adesophageal rows (Fig. 8). Here a single apical organ regenerated between each pair of rows and the adtentacular rows did not regenerate. Although a few of these irregularities occurred on the oral piece following the cutting at each of the four levels of the body, a majority were formed by the oral pieces produced by a cross cut at the bases of the auricles (Fig. 11). The two regenerated apical organs of a single, oral piece were always at first oriented at right angles to the regular one (Figs. 7 and 8). Within a few days, however, these organs began to turn and finally they assumed the regular direction of orientation.



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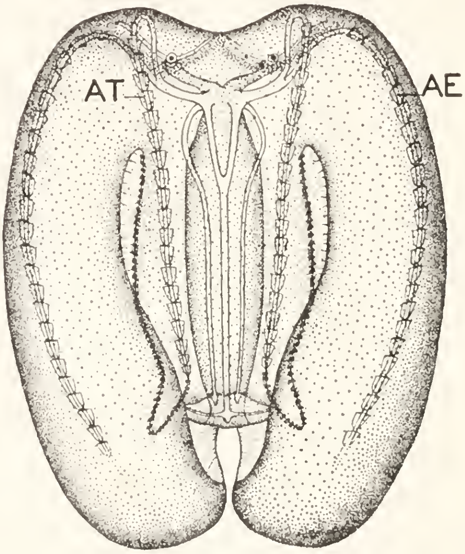

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FIG. 5. This shows the result of grafting two apical pieces together. Each piece represents less than half of an animal. The paragastric and tentacular canals are not shown. This drawing was made on the sixth day.

FIG. 6. This shows the result of grafting together two equal and longitudinal pieces of different animals. Each piece contained slightly more than one half of the body. The drawing was made on the third day.

AE, adesophageal row; *AT,* adtentacular row; *M,* mouth; *ST,* stomodeum.

Experimental Graftings

Several types of graftings were done and since they all have bearing on the problem of regulation and reorganization they are listed and described here under the one heading. A few of these experiments were of an exploratory nature but their results were definite enough to make them significant. Each type of grafting is described here in a single paragraph.

1. Four mid-pieces of separate animals were grafted together with their regular orientation maintained, and the apical and oral ends of a fifth animal were grafted to the apical and oral regions respectively of the fused mid-pieces. By this method of grafting, an animal consisting of the mid-pieces of four animals with the apical and oral ends of a fifth animal was formed. All of the pieces fused at the cut regions and all canals and the stomodeum of each fused in the regular manner. Even the coördination of the plates as well as the feeding reactions at the one mouth were similar to these processes of a normal animal.

2. The apical end of an animal was grafted to the mid-adesophageal surface of another animal. This end fused to the host successfully. Soon after the fusion at the edge of the graft was complete a mouth broke through at one adtentacular region. The canals and rows of the host and the graft fused with each other and the plate movement continued in the regular manner of coördination and direction in each portion of the experimental animal.

3. Two large portions obtained by cutting two animals lengthwise at a slight angle and to one side of the stomodeum were grafted to each other (Figs. 12 and 13). In some the two large portions of animals of an equal size were fused together (Fig. 13) while in other experiments the portions were from animals of unequal size (Fig. 12). There was no apparent difference in the results of the two types of experiments. In all cases the two pieces fused within three days and the corresponding rows fused with each other. This fusion was near the ends of the rows and since two adjacent rows on a normal animal connect to each other there was no irregularity in this fusion. The plate coördination in each piece was quite regular and independent of the other piece.

4. Two pieces were obtained by making a longitudinal cut near the stomodeum and parallel to it through the entire length of the animal. The larger of these two pieces was grafted to another one of similar size and origin. In this graft the orientation of both pieces was unchanged. Fusion took place within three days and the graft continued to live as a single animal. After the operation this organism possessed two apical organs, a single stomodeum, a double number of tentacular

canals, a bifurcated paragastric canal, and a single mouth (Fig. 6). The behavior of this animal was similar to that of any normal one.

5. The two small portions of the animals which were cut in the experiments described in the section above were grafted together. Fusion took place and within six days a single apical organ regenerated and the fused pieces formed a normal animal.

6. The apical pieces of two animals were grafted together at their oral regions so as to form an animal having two opposing apical ends. These two pieces fused and healed very quickly. Although fusion took place, each piece maintained its identity by forming a mouth, by coordinating its own plate movement, and by regenerating auricles and lobes (Fig. 5).

7. Two halves obtained by cutting animals longitudinally through the adtentacular plane were grafted with their orientation reversed. The oral end of one piece was in contact with the apical end of the other piece. Fusion took place immediately and within six days the two halves began to rotate on each other, bringing the similar ends of each piece near each other. This rotation was not carried very far, however, before the specimens died.

8. The apical organ of one animal was grafted to the surface of another animal at various levels of the body. The graft contained some of the infundibulum and the bases of the adradial canals. Fusion took place immediately and the short pieces of the adradial canals connected to each other at first and later they connected to the adtentacular canals

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Explanation of Figures 7–11

Fig. 7. This shows the regenerating end of an oral piece. The oral piece was obtained by making a cross cut through the body at the level of the auricles. Drawn on the fourth day.

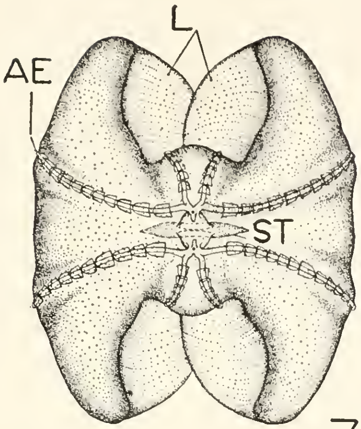
Fig. 8. A drawing of a regenerating oral piece obtained as in Fig. 7. Here the two sets of adesophageal canals are connected to the stomodeum by a separate infundibulum. The adtentacular canals failed to regenerate. Drawn on the fourth day.

Fig. 9. This shows the regular method of regeneration following the removal of an apical piece. This specimen had been cut across at the level of the auricles. The eight rows are formed and the single apical organ is shown in its regular orientation. Drawn on the fourth day.

Fig. 10. This specimen was obtained as in Fig. 9. The result of regeneration failed to bring the canals and rows to the regular type of connection. Drawn on the fourth day.

Fig. 11. This is a diagram to show the distribution of the canals and other organs in an oral piece obtained by cutting the animal across at the bases of the auricles.

AE, adesophageal rows; *F*, infundibulum; *L*, lobe; *P*, paragastric canal; *ST*, stomodeum; *T*, tentacular canal.



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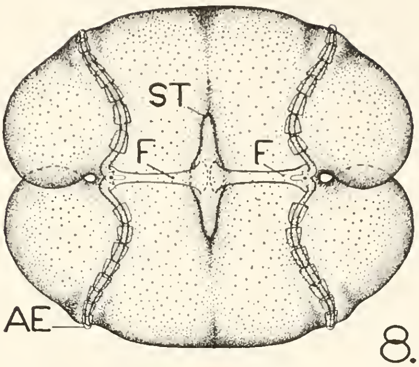

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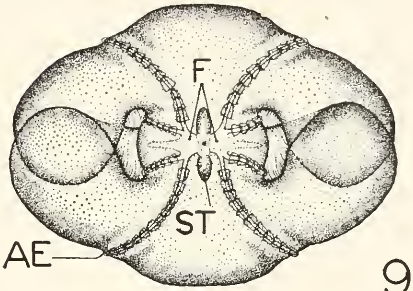

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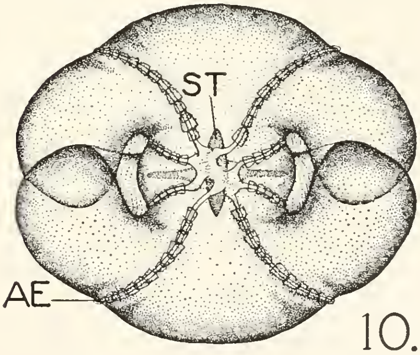

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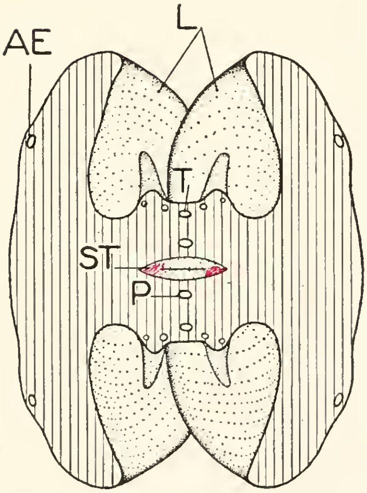

11.

of the host (Fig. 3). The graft always became oriented in a regular manner after its canals joined those of the host regardless of its previous orientation. Later the graft joined the stomodeum of the host (Fig. 3).

9. A small portion of the mid-region of the stomodeum of an animal was grafted to the surface of another animal between its adtentacular rows. Very soon after the healing was complete the stomodeum of the host curved out toward the graft. This bending out toward the graft took place before there was any mechanical connection between the graft

and the stomodeum. Eventually the graft and the stomodeum fused and in addition usually the tentacular canal of the host became connected to the graft (Fig. 2). The graft formed an opening at the surface of the host and thus provided an additional mouth.

DISCUSSION

In considering the problem of symmetry in *Mnemiopsis* it is natural to observe first the anatomy of this animal. The organs are equally distributed along the opposite sides of the main, central, longitudinal axis of its body (Figs. 1 and 11). There are four plate rows, two auricles, one paragastric canal, one tentacular canal, and one lobe on each side of the stomodeum. The apical organ and the mouth are at the two poles of the stomodeum and they lie in a plane at right angles to it. Then as far as the organs, with the exception of the apical organ and the mouth, are concerned they are distributed in the body bilaterally about the stomodeum. Therefore this distribution of organs indicates a bilateral symmetry without a dorso-ventrality.

Does *Mnemiopsis* have any features other than its anatomy which might give some information about its symmetry? In answering this question I shall consider certain observations made on experiments in this animal. The outstanding feature of this animal during regeneration

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EXPLANATION OF PLATE I

FIG. 12. This shows two longitudinal pieces from animals of different sizes fused together. This fusion was complete by the end of four days.

FIG. 13. This is a photograph of the fused two longitudinal pieces from animals of equal sizes. The fusion was complete by the end of the fourth day.

FIG. 14. An apical view of an oral piece obtained by cutting an animal across through its infundibulum. This shows the irregular row connection and two apical organs have regenerated. Photographed on the third day.

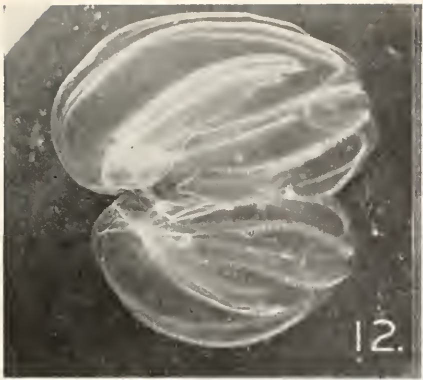
FIG. 15. An apical view of an oral piece obtained as shown in Fig. 14. This shows the regular row connection and but a single apical organ has regenerated. Photographed on the third day.

FIG. 16. An apical view of an oral piece obtained by cutting an animal across at the auricular region. This shows the irregular row connection and two apical organs have regenerated. Photographed on the third day.

FIG. 17. An apical view of an oral piece obtained as is shown in Fig. 16. This shows the usual row connection with but one apical organ regenerated. Photographed on the third day.

FIG. 18. This shows the formation of a single bulb at the apical end of the stomodeum. This follows the cutting off of the apical portion of the animal. The rows which are not continuous at the top of the photograph were broken during the handling of the specimen.

FIG. 19. An apical view of an oral piece obtained by making a cross cut at the region of the auricles. This shows the usual connections of the rows and two apical organs have been formed during regeneration. Photographed on the third day.



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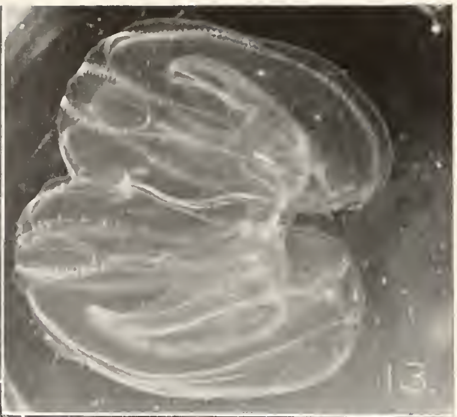

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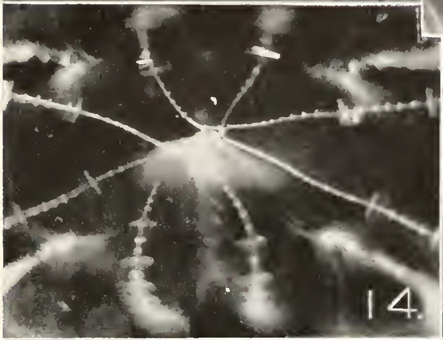

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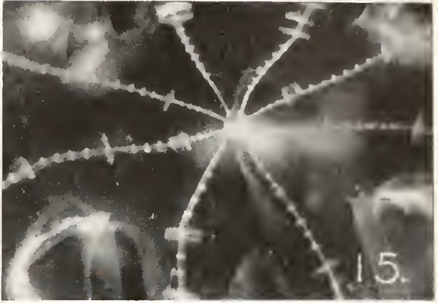

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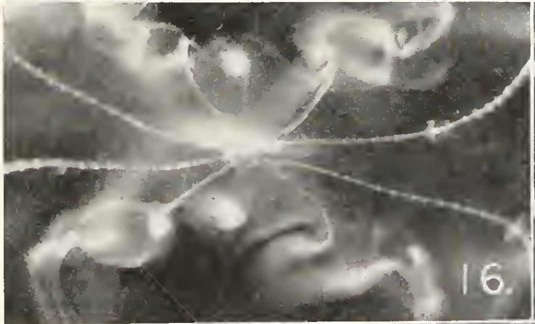

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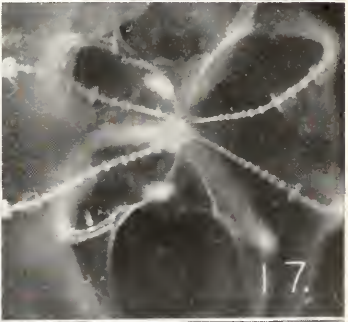

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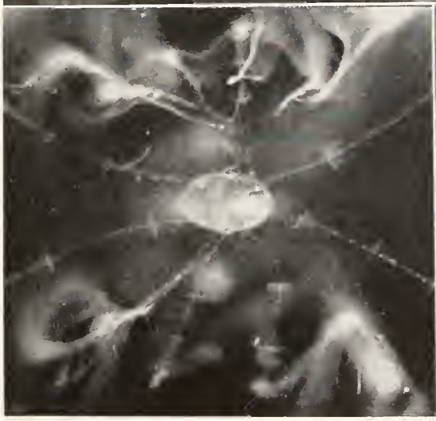

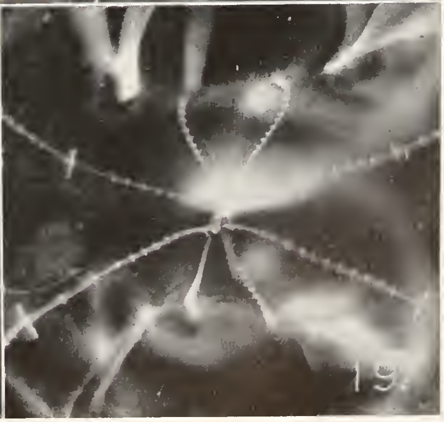

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PLATE I

is its ability to regulate its own reorganization following any type of disturbance to its body that I have made. This power to regulate has been remarkably demonstrated during the process of regeneration. When this animal has been cut into halves, thirds, or fourths either by cross cuts or by longitudinal cuts the removed parts were regenerated perfectly with but few exceptions (Coonfield, 1936*a*). Following the cutting of its body across at any level above the mouth the procedure of regeneration showed a bilateral arrangement with but few exceptions. The apical end of the stomodeum formed a bulb (Fig. 18), then this bulb split in two (Fig. 4). Later these bulbs thinned out and formed the two radial canals. Both the tentacular and the paragastric canals established their regular connections with these two bulbs (Fig. 4) and as these bulbs disappeared as such these canals became connected to the base of the infundibulum (Fig. 2). In the meantime the apical end of the stomodeum became elongated and formed the infundibulum with a single apical organ formed on the end of this structure. During the regeneration of the apical organ the plate rows were regular in their reformation. Two adjacent adtentacular rows and a single adesophageal row on either side of them became connected to each other at first (Fig. 9). Then as the bulbs became the radial canals the plate rows established their usual connection to the infundibulum by way of the radial canals. The regular organization was complete after the apical organ had regenerated.

How can the irregularities in regeneration be accounted for even though they are relatively few in number? These irregularities have appeared following the cutting of the animal into halves, thirds, and fourths (Coonfield, 1936*a*). A very few of the regenerating pieces failed to reform the lost plate rows. A few irregularities are given in the present report. These are concerned with the failure of the rows to become connected in the regular manner (Figs. 10, 14 and 16), or with the regeneration of two apical organs instead of a single one (Figs. 7, 8, 14, 16 and 19), or with a considerable splitting of the stomodeum to form two infundibula (Fig. 8). Even though I cannot offer a satisfactory reason for the appearance of these irregularities I believe they are significant in supporting the view of bilateral symmetry. For in all except three or four that failed to reorganize the plate rows in the regular manner the reforming organs were distributed according to bilaterality. So then the regulation of the reforming organs in the body of *Mnemiopsis* whether they proceeded in the regular manner or exhibited some irregularities supports the view of bilateral symmetry.

Information having a direct bearing on the problem of symmetry and regulation in addition to that shown by a study of the anatomy of *Mne-*

miopsis and observations on regeneration in this animal was obtained by performing experiments involving grafts. This animal showed a remarkable ability to regulate itself both according to structure and physiology in response to this type of experiment. The ability of this animal to organize the grafts in experiments wherein a whole body was formed by fusing the mid-pieces of four animals with the apical and oral pieces of a fifth animal, the noninterruption of the regular activities of both the graft and the host following the fusing of an apical piece at right angles to the surface of the host, and also wherein a large portion of two animals were fused together (Figs. 12 and 13) show the ability of this animal to regulate itself. The reorganization following the fusion of two longitudinal parts of the body (Fig. 6) shows that the animal almost succeeded in organizing a normal body. This opinion is confirmed by noting that the two parts of the stomodeum fused as one and with but one mouth, the two paragastric canals on each side of the stomodeum united all along except near the infundibulum, and the actions of this animal were similar to those of a normal one. In the experiments whereby two apical pieces were fused to each other at their oral regions the ability of each piece to regulate itself although being fused to another similar one was definitely shown.

Further evidence supporting the view of regulation in a physiological manner was obtained by observing polarity in *Mnemiopsis* following experiments involving grafts. Polarity has been observed previously in this animal (Coonfield, 1934 and 1936*b*). The data contained in the present report show that when two halves of an animal were grafted together with their orientation reversed each piece attempted to rotate so as to bring the two similar regions together. Also when an apical organ or a piece of the stomodeum was grafted to the surface of an animal these grafts always became oriented and established connections to the plate rows or the other canals, and to the stomodeum (Fig. 3). It is interesting to note that the apical organ became oriented only after connections to the canals had been established and both the canals and the stomodeum were attracted to the stomodeal grafts immediately after each had become healed in the body of the host. The apical grafts persisted long enough to show that their form and function were unchanged by their new location. Also the stomodeal grafts which were taken from the side of the stomodeum not only continued to function as a stomodeum but in addition each formed a mouth. In summing up the data obtained from observing the various types of grafts which were carried out on *Mnemiopsis,* it seems to me that these data show that regulation is demonstrated by them in this animal. Since regulation in this animal can be associated with symmetry, it seems that these experiments support the view of bilaterality without dorso-ventrality.

CONCLUSIONS

1. *Mnemiopsis* possesses a bilateral symmetry without a dorso-ventrality. This is shown by the anatomy of this animal and by its method of regulation following a disturbance to its body.

2. Symmetry and regulation are two closely associated features of *Mnemiopsis.*

3. Regulation is demonstrated in *Mnemiopsis* by the following results.

a. Reorganization during regeneration which follows the cutting of its body.

b. Reorganization during the fusion, orientation, and connections of grafts.

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ON THE SIGNIFICANCE OF THE POLAR SPOT IN RIPE UNFERTILIZED AND IN FERTILIZED ASCIDIAN EGGS

A. DALCQ (Brussels) AND G. VANDEBROEK (Ghent)

Cohen and Berrill (1936) have given an interesting account of observations on eggs of *Ascidiella aspersa* and *Phallusia mamillata.* After the jelly was digested by the gastric juice of the crab *Munida,* the eggs were stained *in toto* with a vital dye (particularly Nile-blue sulfate) and subsequent events studied with special reference to changes in the form of the germ and in the appearance of the vital dye within the egg. These are described in the ripe egg, from the time of fertilization until the formation of the polar bodies, and during cleavage, gastrulation, and formation of the tadpole.

In the ripe unfertilized egg they have observed the "polar pit" ("tache polaire" of *Ascidiella*—Dalcq, 1932) though they question whether this cortical differentiation marks the position of the maturation spindle and identifies the location of the animal pole. Their doubt is not based on continuous observation of the region of the polar pit from which the dye entirely disappears before the elevation of the polar body, nor have they been able to determine directly whether the polar body is actually formed in the polar pit or not. They are influenced, however, by certain cases in which foreign particles adhere to the cortical layer of naked eggs in the vicinity of the polar pit. On measuring the distance between such a particle and the pit, and, later, between the same particle and the polar body, they found considerable variation in the distances. In two cases, drawings of which are given in their paper, the distance was increased; the same was true of "several" other eggs. In one case the distance did not change. While Cohen and Berrill consider the possibility of a displacement of the marking particle by some movement of the cortical layer, they discard such an explanation on the following grounds. During the maturation period the egg shows, grossly, only a flattening and an elongation, while the deformation during cleavage, though much greater, is unaccompanied by any change in the distance between the polar body and the adhering particle. Hence they do not believe it possible that the earlier deformations of the egg could be responsible for the displacement of the small body fixed to the cortex. They feel justified in stating, therefore: "Since Dalcq accepted the site of the polar pit as being identical with that of the polar body and used it to orient the eggs for cutting, it follows that some doubt is thrown on the validity of his conclusions regarding the localization of presumptive germinal regions in the fertilized egg of *Ascidiella aspersa*, although, of course, not on the existence of such presumptive regions" (p. 84). They do not state what, in their opinion, the significance of the polar pit is. On the basis of experiments cited below we have good reason to believe that their conclusion is not justified.

It is unfortunate that Cohen and Berrill have discarded the possible explanation of the behavior of the attached particles in their experiments on the basis of cortical movements. Such movements are common in eggs and may be easily identified by means of definitely localized vitally stained areas which may be followed through ensuing stages. This method was used by one of us (G. V.) at Roscoff[1] during the summer of 1935 with outstanding success. We shall mention here only the results necessary to dispel the doubt thrown by Cohen and Berrill on the significance of the polar "pit," perhaps better called the polar "spot." In addition, we shall describe briefly an anomaly observed by the senior author (A. D.) which gives further proof of the existence of a maturation spindle immediately under the polar spot.

The ripe egg of *Ascidiella*[2] contains the first maturation spindle, as stated by Cohen and Berrill, and not the second, as previously stated by Dalcq on the basis of the examination of unfertilized eggs and the appearance of the spindle in sectioned preparations. On continuous observation two polar bodies may be seen to be successively extruded by the egg after fertilization. The jelly of the egg was removed with the aid of mounted needles (watchmakers' forceps can also be used), some dozens of naked eggs easily being prepared in but a few minutes. The method is not tedious.

In the discussion of merogony experiments Dalcq has already called attention to the fact that after insemination of naked eggs, the penetration of the spermatozoön into the egg may be delayed by as much as half an hour. Vandebroek has more recently discovered, in the course of experiments which will be reported in detail elsewhere, that the moment of sperm penetration can be recognized by a series of characteristic deformations of the egg which persist for about two minutes. The egg then returns to the spherical condition until the time of first polar body formation. As is noted below, the first and second polar bodies are formed 6 and 20 minutes respectively after the penetration of the spermatozoön. The actual times vary with temperature but the above are those observed in the present experiments. In dealing with eggs in the brief period preceding maturation it is especially important to recognize the delay mentioned above and to identify the moment of sperm penetration. This, unfortunately, Cohen and Berrill have failed to do.

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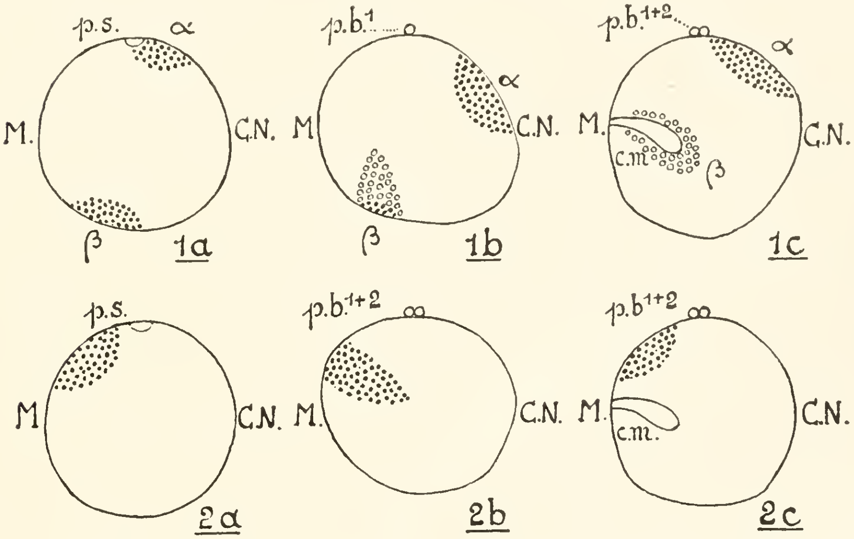
[1] We wish to express our gratitude to Professor Dr. Ch. Pérez and Dr. G. Teissier for the excellent working opportunities accorded both of us during several stays at the "Laboratoire Lacaze-Duthiers."

[2] Because of some confusion as to the identification of the species used, it is necessary to state that our experiments were made on the *Ascidiella* which is found in abundance in the great pool of the Roscoff Laboratory, and which was used by Dalcq and by Tung in 1931 and 1932, and called by them *Ascidiella aspersa*. Berrill (1928) suggests that, because of the small size of the animal and the fact that the eggs do not float, the Roscoff species should be called *Ascidiella scabra*, the name of *Ascidiella aspersa* being reserved for the great *Ascidiella*, with floating eggs, common in Plymouth (and in the vicinity of Boulogne). Dalcq and Tung, following this suggestion, used the name *Ascidiella scabra* in their subsequent publications.



FIGS. 1 AND 2. Three views of eggs subjected to local vital staining: (*a*) ripe egg; (*b*) after the formation of the first polocyte; (*c*) during the formation of the yellow crescent and hyaline plasma zone. Colored territories stippled in deep black, when superficial invaginated parts (Figs. 1*b* and *c*) marked with ooo; *p.s.*: polar spot; pb^1: first polocyte; pb^{1+2}: the two polocytes; *M.*: mesoblastic side; *C.N.*: chordoneural side; *c.m.*: mesoblastic region (yellow crescent). The two eggs are viewed from their left sides.

In the experiments reported below, localized areas of denuded ripe unfertilized eggs were vitally stained with the dye 'Brillant Cresyl.' The stained areas have a diameter of from 50 to 75 μ (ca) and persist, perfectly localized, during the hours immediately following fertilization. If the egg is not fertilized the dye rapidly diffuses. Three experiments which demonstrate the significance of the polar spot will be described.

In the first case (Figs. 1*a*, 1*b*, and 1*c*) one mark (α) was made in the immediate vicinity of the polar spot and another (β) on the vegetative pole (Fig. 1*a*). In the second case one mark only was made, near the polar spot (Fig. 2*a*). In the third egg the mark was located a short distance from the pole, between the polar spot and the equator (Figs. 3*a* and 3*a*′). Tracing the fate of mark α of the first egg, we see that two minutes after fertilization the angular distance between the polar spot and the adjacent border of the colored region is increased. Comparing this with the similar behavior in the other eggs, it may be noted that all of the marked areas on the animal hemisphere have increased their distance from the polar end by about 30° (Figs. 1*b*, 2*b*, 3*b*, and 3*b*′). They maintain these positions in spite of the considerable deformations which the egg undergoes during the period of expulsion of the two polar bodies. In the case illustrated by Fig. 1, the presence of the mark enables us to determine that the first polar body is actually formed in the polar spot about 6 minutes after fertilization. The second polar body is formed next to the first about 14 minutes later (Figs. 1*b* and 1*c*). It appears, therefore, that the polar spot is also the maturation site.

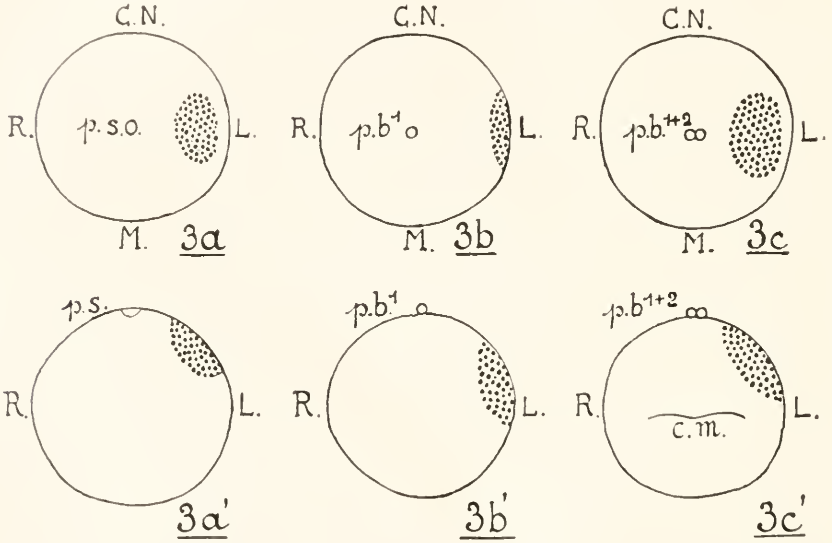


FIG. 3. An egg with lateral coloration of the animal pole. *a*, *b*, *c*: viewed from the animal pole; *a*′, *b*′, *c*′: viewed from the mesoblastic side. Other abbreviations as in Figs. 1 and 2.

In the post-maturation phases during which the female pronucleus migrates toward the center of the egg to meet the male pronucleus and the yellow crescent is gradually formed, the behavior of the marks in the vegetative pole is very interesting. This is to be described in detail elsewhere (by G. V.) but is suggested by the mark β of case 1. In the present consideration we are directly concerned with marks on the animal half of the egg and these consistently show a movement toward the animal pole which results in their return to their original positions (Figs. 1*c*, 2*c*, 3*c*, and 3*c*′). They show no further change during the cleavages which follow.

The appearance of the yellow crescent and the adjacent hyaline protoplasm which contains the fusion nucleus allows one to determine definitely the orientation of the egg. Using these landmarks, it was found that in the egg of Fig. 1, the mark α was in the plane of symmetry, just above the chordoneural material, while the mark β lay under the future yellow crescent, i.e. on the mesoblastic side of the germ. In the second egg the mark had been put between the polar spot and the presumptive mesoblastic crescent, nearly in the plane of symmetry. In the third case, on the other hand, the mark was located in a region to one side, in the left half of the germ. In these three typical experiments, therefore, the chief regions around the polar spot have also been explored.

To summarize briefly, it has been found that, after fertilization, the cortical layer of the ascidian egg undergoes definite displacements. During the initial period of active deformations, the cortical layer surrounding the animal pole moves toward the equator. Later, when the conjugation of the pronuclei takes place, the same material is again shifted nearer to the animal pole. In conclusion, it may be stated without the least hesitation that the polar spot indicates the site of formation of the polar bodies, and is the result of the presence of the first maturation spindle just below the cortex.

In view of these conclusions the question now arises as to how the data of Cohen and Berrill are to be interpreted. It seems clear that the variations they record in the distance between the adhering particle and the animal pole result from the activity of the cortical film; the cases in which the distance did not change may be explained on the assumption that the eggs were first observed somewhat too late. This explanation is not entirely satisfactory, however, for they appear to have observed some of the eggs continuously and yet fail to record any secondary shift of the materials towards the animal pole. In this connection we should like to make several suggestions inasmuch as it must be admitted that observations made by means of adhering particles are less reliable than those based on local vital staining of parts of the egg itself. The possibility of a displacement of the particle during the later deformations of the egg should be examined. It is possible that the particle might eventually penetrate the cortical film and adhere directly to the underlying granular cytoplasm.

For the latter form, the morphological value of the polar spot is now established beyond any doubt and it would appear that Dalcq was justified in basing his merogony experiments on this indication of the animal-vegetative axis of the egg. Sectioning of the ripe ascidian egg in various planes relative to this primary axis and subsequent fertilization of both pieces seems to be at present the sole method of analysing the distribution of the potencies at that stage. Further experiments have been carried on by the senior author during recent years and a short account of the results has been presented (Dalcq, 1935). This has led to a further cytological study of the structure of the ripe egg, and to the discovery of a preëxisting bilateral symmetry indicated by the arrangement of eosinophil subcortical granules (the material of the future yellow crescent) in the form of a large crescent which is more developed on one side of the primary axis. This important feature of the organization of the ripe *Ascidiella* egg together with the variations in the structure of merogonic twin embryos, whose anatomy has been thor-

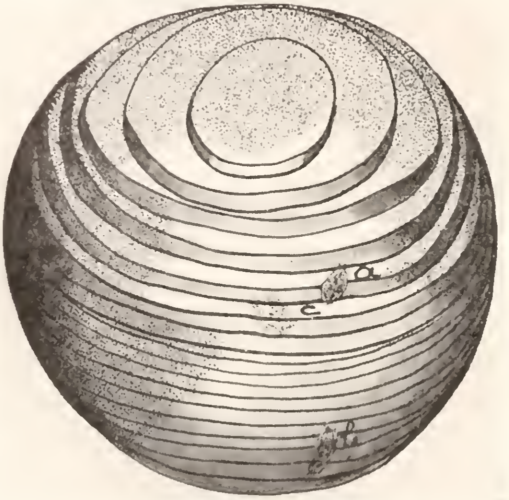


FIG. 4. Reconstruction of a fixed abnormal *Ascidiella* egg. Fixation, Allen; Sections, $6\,\mu$; Staining, Hollande. Perspective view at 45° (method of Lison). *a, b, c:* the three maturation spindles. The eosinophil subcortical material is stippled.

oughly studied in a quantitative way, with several graphic reconstructions, will be fully described in another place.

In relation to the problem of the significance of the polar spot considered here, it seems important to draw attention to an anomaly which has to be borne in mind when performing merogony experiments. Among numerous sections of ripe eggs, the senior author has observed, four or five times, eggs which appear to possess two polar spots. Such eggs were at first discarded without further consideration, but later, a similar case was fixed and sectioned. The slide shows that the egg contains no less than three maturation spindles. In Fig. 4 may be seen a graphical reconstruction of the egg by Lison's method (1936). The three spindles lie in the region free from the eosinophil granules which nearly cover the vegetative hemisphere. The three spindles are small, of approximately equal size, and each supports some chromosomes. Owing to the direction of the plane of sectioning, which was more or less parallel to the primary axis of the egg, two of the spindles (*a* and *b*) lie under the part of the cortex toward the observer of the drawing; the third *c*, is situated on the opposite side, not so far from *b* as it appears to be on the drawing.

The infrequent occurrence of such eggs is of some importance to the investigator who is performing merogony experiments and throws some light on the significance of the polar spot. It is clear that the existence of a secondary spot corresponds here to a supplementary spindle. Why two and not three spots were seen may be explained in either of two ways; either one of the spots escaped the eye of the observer, or one of the spindles did not adhere sufficiently to the cortex to be seen *in vivo*. No positive information has been obtained concerning the origin of such anomalies. It seems probable that the spindle material became divided when the rupture of the germinal vesicle took place.

SUMMARY

Ripe *Ascidiella* eggs have been subjected to local vital staining, chiefly in the regions surrounding the animal pole. Fertilization is immediately followed by a shift of the cortical layer towards the equator; when the yellow crescent appears, the material returns to its original position. The continuous observation of the ripe and the fertilized egg shows, contra Cohen and Berrill, that the polar spot marks the place where polocytes will be extruded.

In addition, attention is called to cases where there are two or three maturation spindles in the same egg.

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REPRODUCTIVE SYSTEM AND COPULATION IN AMPHISCOLOPS LANGERHANSI (TURBELLARIA ACOELA)

LIBBIE H. HYMAN

(From the Bermuda Biological Station for Research and the American Museum of Natural History)

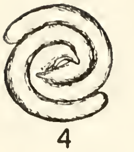
The Turbellaria Acoela constitute one of the most interesting invertebrate groups because they are the lowest of the bilateral animals (if the Mesozoa be excepted). Their simplicity of structure is in all probability primitive and not the consequence of degradation. Among their primitive characters are the absence of a definite digestive entoderm, the presence of a statocyst, the netlike nervous system with radially arranged principal nerve strands, the scattered sex cells, the absence of female ducts, and the simple construction of the copulatory mechanism. In the Acoela the entoderm has apparently not yet differentiated from the general mesentodermal cells. In the most primitive genus, *Nemertoderma,* a definite brain is lacking, the nervous system consists of a subepidermal plexus, and the reproductive system is limited to the sex cells, as ducts and copulatory apparatus are entirely wanting (Steinböck, 1931). The extremely complicated reproductive system characteristic of the Platyhelminthes has evidently arisen within the phylum itself, starting from conditions in the Acoela, where this system may not have advanced beyond the stage seen in cœlenterates, as in *Nemertoderma.* The first step in the development of a reproductive system among the Acoela consists in the formation of more or less definite paired channels in the parenchyma for the transport of the sperm. These somewhat vague vasa deferentia unite to a common terminal duct in whose wall a penis gradually evolves, first as a muscular thickening of the wall, then in most Acoela as a conical penis composed of parenchyma, muscle fibers, and in some cases gland cells also. Female ducts are absent throughout the group but in the majority there is a seminal receptacle, the seminal bursa, in the form of a rounded parenchymatous organ which may or may not be connected to the exterior either by its own pore or by way of the male duct and pore. This seminal bursa has no direct connection with the eggs but possesses a variable number of projecting exits, composed of a hard material arranged in rings. These exits were termed "mouthpieces" by Mark (1892) and his name has been adopted by German workers in the form Mundstück, but they so much suggest the nozzle of a hose that I shall call them nozzles. In some Acoela there are numerous seminal bursæ each with one nozzle.

It is thus evident that most of the Acoela possess a copulatory mechanism in the form of a protrusible penis papilla and a seminal receptacle but to my knowledge copulation has never been recorded for the group. The only account in the literature which I have been able to find is that of Gardiner (1898), for *Polychœrus caudatus.* Gardiner noticed that restless individuals often mounted on quiescent ones and remained in this position for a brief period. Sections of the quiescent worms showed sperm on the surface and in the parenchyma and Gardiner therefore concluded that in this species fertilization occurs by means of hypodermic impregnation. While hypodermic impregnation is probably of wide occurrence in the Acoela it is to be presumed that a true copulation also takes place since the necessary mechanism is present, as von Graff has pointed out (1908).

During a stay at the Bermuda Biological Station in the summer of 1935 I had ample opportunity to witness the breeding habits of an acoel present in the aquaria there. After careful study of living intact and pressed specimens and serial sections, I have identified this animal as *Amphiscolops langerhansi* (Graff) 1882. Specimens agree in all details with von Graff's 1904 description. This species has flourished and bred for some time in the aquaria at the Bermuda station but its source is unknown. Dr. Wheeler, the Director, informed me that the animal had not been found in nature at Bermuda although I collected a very closely related form from the sea-weeds near the station. The species has apparently been introduced into the aquaria from some outside source. It is recorded by von Graff from Madeira, the Canary Islands, and the Mediterranean.

Amphiscolops langerhansi (Fig. 1) is a small worm, reaching 4–5 mm. in length, and of elongated form with a bilobed posterior end. Near the anterior end is a conspicuous statocyst and two small eyes. The brown color is due to numerous zoöxanthellæ situated in the peripheral part of the parenchyma. A study of their rôle in the life of the animal has recently been made by M. F. Welsh (1936). In addition there occur just under the epidermis areas formed of an apparently crystalline material, called concrement crystals by von Graff; they are dark by transmitted, opaque white by reflected light and confer a pattern on the animal. This pattern differs considerably in different individuals but its general arrangement is indicated in Fig. 1. The mouth is central and near the posterior end can be noticed the rounded mass of the penis.

Although the reproductive system of *A. langerhansi* has been ade-



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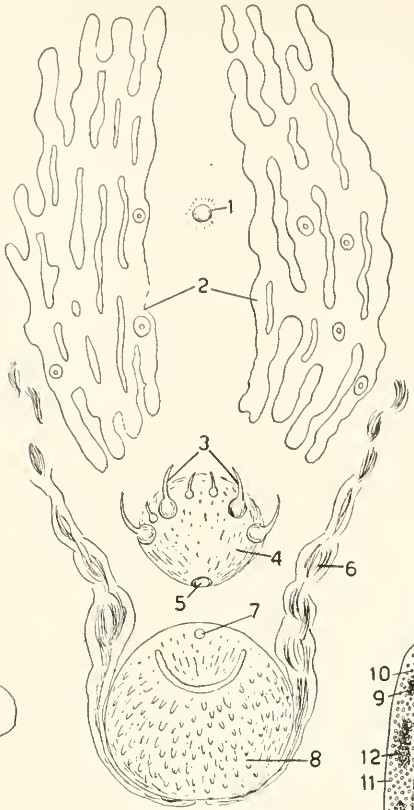

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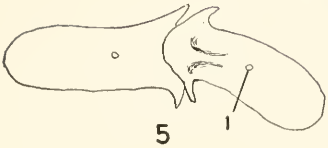

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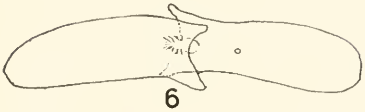

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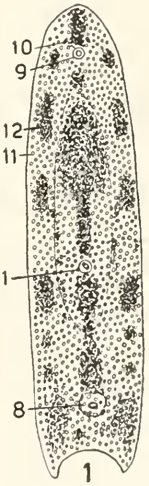

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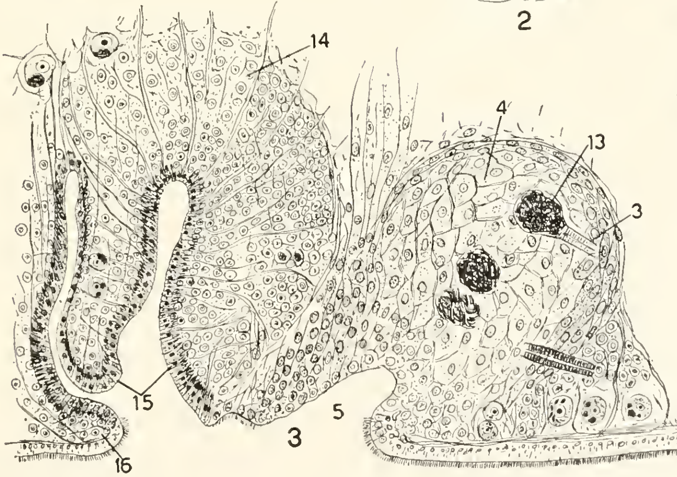

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FIG. 1. *Amphiscolops langerhansi,* from life, showing the pattern of concrement granules.

FIG. 2. General view of the reproductive system in a living pressed specimen.

FIG. 3. Penis and seminal bursa in sagittal section.

FIG. 4. Beginning of copulation, rolled-up stage.

FIG. 5. Attitude during copulation.

FIG. 6. Copulating pair in motion, right-hand individual advancing and dragging left-hand one with its dorsal surface against the glass.

quately described and figured by von Graff (1904), it is necessary to review its main features in order to understand the copulatory process. Its general appearance as seen in a live worm pressed under a cover-glass is shown in Fig. 2. The ovaries consist of strands of eggs in a paired lateral location. Behind and unconnected with the eggs lies the median rounded seminal bursa, or sperm receptacle. This bears several anteriorly directed nozzles. The number of these is variable and increases with age so that small ones occur along with large ones in the larger specimens. Von Graff records finding 6–11 nozzles and I have found 2–8, usually 4 large ones and one or more small ones. These nozzles consist of a hard yellow material, commonly spoken of as chitinous; but no proof exists of the occurrence of chitin in the Platyhelminthes and they are probably cuticular in nature, that is, composed of a scleroprotein allied to keratin. The nozzles are cross-striated and appear to consist of a series of superimposed rings. They are pointed towards the ovaries and serve to discharge sperm against the eggs. The end of the nozzle buried in the tissue of the bursa connects with a rounded sac filled with sperm. The bursa opens below by the female genital pore. Immediately behind the bursa lies the large rounded mass of the penis filled with gland cells. To either side a somewhat indefinite channel, the vas deferens, containing masses of sperm, approaches the penis, passes around to its posterior side and there opens into the penis base. The testes consist of cell groups scattered throughout the lateral parenchyma.

For an adequate understanding of bursa and penis, sections are necessary. This region of the animal is illustrated in Fig. 5, a median sagittal section. The seminal bursa is composed of loose reticular fibers in the meshes of which parenchyma cells occur. It has no definite cavity but there are many small spaces and several larger spaces each occupied by a dense mass of sperm. From each such sperm mass a nozzle extends anteriorly. Posteriorly and ventrally the bursa opens into the female genital pore by way of a small invagination which is not lined by a definite epithelium.

The penis considerably resembles that of triclads but is of a looser, less muscular construction. It consists of a loose mass of parenchymal and gland cells, not well differentiated from each other, through which run radiating muscle fibers. These muscle fibers join the general subepidermal muscle layers. As in triclads, the penis is divisible into an internal mass, the penis bulb, continuous with the general parenchyma, and the projecting papilla. This papilla is continuous anteriorly with the parenchyma of the seminal bursa; it is free only posteriorly, where it is separated by a deep cleft from the succeeding part of the body.

The body wall behind this cleft forms an anterior projection which unexpectedly has been found to play an important rôle in copulation. I shall therefore call it the sperm guide. Unlike the rest of the body surface, which has no definite epithelium (the epidermal cells are "eingesenkt" as the Germans say) the penis lumen and cleft separating penis papilla from the sperm guide are lined by a more or less definite epithelium. The cavity in which the penis papilla lies opens ventrally by the male pore which is so close behind the female pore that the two may be considered with von Graff to form a common shallow space.

In one aquarium at Bermuda which contained five or six hundred specimens of *Amphiscolops,* copulation was observed on numerous occasions. At all daylight hours from 6 A.M. to 5 or 6 P.M., one or two to a dozen pairs in copulation could be seen on the wall of the aquarium facing the light. Apparently no copulations occur after dark and usually the number decreased after 4 P.M. As the number of sexually ripe individuals in the tank was estimated at less than two hundred and as fifty or more copulating pairs were counted daily, it is evident than any one animal must copulate repeatedly. To test this, five copulating pairs were isolated into a finger bowl. The following day, two egg masses were found in the bowl and one pair was again in copulation.

Copulation occurs in the following manner. When two individuals wandering on the aquarium wall happen to come in contact they generally give each other quick little touches resembling nips with the anterior end. This behavior commonly occurs whether copulation ensues or not. Only the larger specimens copulate. Often a large specimen was seen attempting copulation with a smaller one which declined the invitation. If both individuals are ripe for copulation, they, following a short period of the nips just mentioned, suddenly roll up into a ball (Fig. 4), with one individual on the outside. The posterior end of this individual is so curved that its ventral surface is in contact with the ventral surface of the tail end of the other copulant. After a very short interval, less than thirty seconds, the two worms unroll and are seen to be in firm connection. The upper individual simply flattens its ventral surface against the glass and becomes quiescent. The other one is compelled to twist about until its ventral surface touches the glass. Both then become motionless with ventral surfaces against the glass and heads pointed more or less directly away from each other (Fig. 5). If disturbed they will crawl about, one animal with ventral surface against the substratum dragging the other, dorsal side next the substratum, after it (Fig. 6). The union is thus very firm, and the copulatory act is prolonged. In a number of undisturbed pairs which were timed connection lasted from forty to fifty-five minutes, usually about fifty minutes. The animals then quickly separate, each gives a comical little shake, as if settling its viscera into place, and proceeds about its business.

The process of copulation connot be understood by a mere inspection of copulating pairs. Indeed, study of these with a high-power hand-lens was puzzling rather than informative for the location of the connection indicated that the penis papilla was not inserted into the seminal bursa. The natural assumption, of course, is that the penis papilla discharges directly into the bursa and a statement to this effect is made by Bresslau (1933, p. 118). Inspection of Fig. 3 shows, however, that the seminal bursa has no definite lumen and that the female

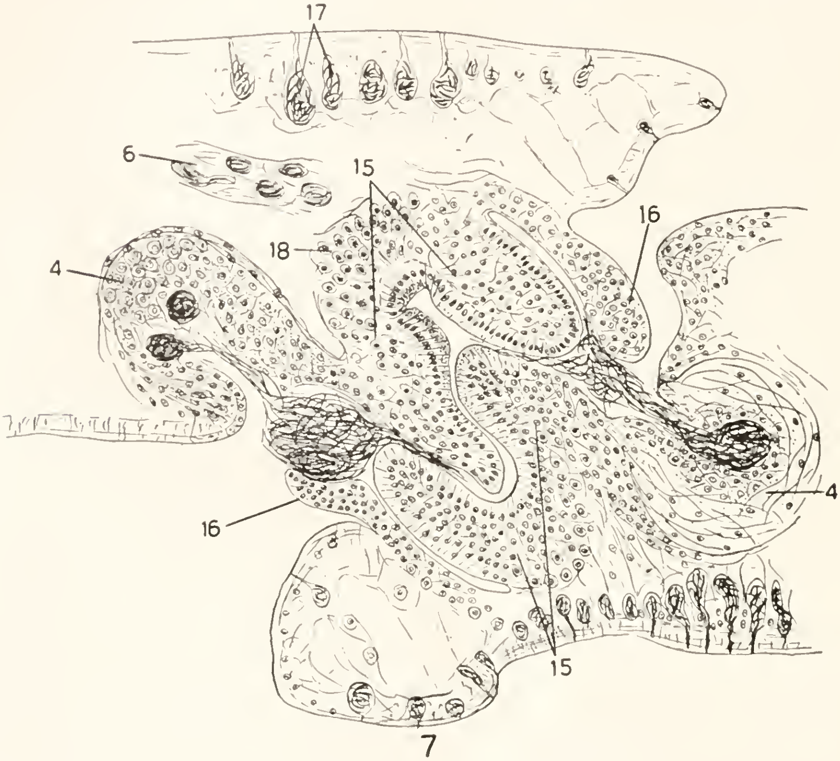

FIG. 7. Section through a copulating pair, reconstructed from several sections, showing manner of interlocking of the penes and stream of sperm, directed by the sperm guide, flowing into the seminal bursa of each copulant.

In all figures: 1. mouth, 2, ovary, 3, nozzles of seminal bursa, 4, seminal bursa, 5, female genital pore, 6, vas deferens, 7, male genital pore, 8, penis, 9, statocyst, 10, eye, 11, zoöxanthellæ, 12, area of concrement granules, 13, sperm mass in bursa, 14, penis bulb, 15, penis papilla, 16, sperm guide, 17, cutaneous glands, 18, gland cells of penis bulb.

genital pore is too small and shallow to receive the penis. Fortunately it is easily possible to fix pairs in copulation and several such have been studied in serial sections.

A sagittal section of a copulating pair is drawn in Fig. 7. It is seen that not only the penis papilla but also the sperm guide is protruded and that the penes are interlocked in a dovetailed fashion such that the lumen of each penis grasps the posterior part of the penis of the partner. This leaves the anterior part and sperm guide of each copulant directed towards the bursa of the other. The sperm issue in a stream from the penis lumen and pass over the anterior edge of the partner's penis towards the latter's bursa. They are prevented from escaping into the sea-water by the sperm guide which directs them towards the bursa. They enter the bursa and pass into the sacs connected with the nozzles. Study of serial sections of the bursæ of copulating animals has shown that the sperm easily penetrate throughout the bursa by way of spaces and so the one stream of sperm reaches all of the sperm sacs in the bursa. It is evident that some glandular secretion accompanies the sperm for the lining epithelium of the proximal part of the penis lumen contains secretion granules and a secretion staining with hæmatoxylin and thus probably albuminous in nature also is found in the penis lumen.

The copulation is thus seen to be mutual as in the Platyhelminthes in general, each worm fertilizing the other. The manner of copulation is, however, unique, consisting of an interlocking of the penes. The only comparable case that occurs to me is that of the slugs where in many species the penes twine about each other in copulation and deposit a sperm mass upon each other.

The eggs are laid at night, probably after midnight, in flat gelatinous cakes stuck to the aquarium glass or other objects. The material in which the eggs are imbedded is probably secreted by the very numerous cutaneous glands. It is evident from anatomical considerations that the eggs cannot be laid by way of the female genital pore. They probably issue through the mouth but unfortunately the process was not observed. The number of eggs in the egg mass was counted in 44 cases and found to range from 3 to 16, with the majority containing 6 to 9 eggs. The eggs hatch on the fifth day into completely formed little worms which, however, do not possess any zoöxanthellæ.

Early in the morning, about 6 A.M., the eggs laid during the night are found for the most part in 16- to 32-cell stages but a few earlier cleavages can generally be discovered. The early cleavages were watched in a number of eggs and did not seem to accord with the standard account, derived from the work of Bresslau on *Convoluta* (1909, 1933). The first cleavage was sometimes equal, sometimes unequal. A three-cell stage was common and the four-cell stage showed no definite arrangement into two micromeres and two macromeres. The cleavage of the four-cell stage was not followed satisfactorily owing to the sudden shifts in position occurring at the moment of cleavage. The time remaining after the animals began to lay eggs was too brief to permit any extended study of the cleavage, but a number of egg masses were preserved and may be studied at some future time.

For the privileges of a stay at the Bermuda Biological Station I am greatly indebted to the President, Professor E. G. Conklin, and the Director, Dr. J. F. G. Wheeler.

Summary

In the acoelous turbellarian *Amphiscolops langerhansi*, copulation occurs by the interlocking of the penes. The sperm pass over the anterior surface of the partner's penis into the seminal bursa of the latter. The body region immediately behind the penis is also protruded in copulation and acts as a sperm guide directing the sperm into the partner's bursa and preventing their dissemination into the water.

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A NEW GENUS OF HYDROID AND ITS METHOD OF ASEXUAL REPRODUCTION

SAMUEL STOCKTON MILES

(*From the Mount Desert Island Biological Laboratory*)

A tubularian hydroid (Plate I) was found in the waters of Frenchman's Bay off Salsbury Cove, Mt. Desert Island, which apparently is a hitherto undescribed genus. It is a solitary form dredged off mud bottom at about 40 to 60 feet along with *Corymorpha pendula* and *Acaulis primarius.* Many specimens were found. Its peculiar interest lies in an unusual mode of asexual reproduction.

The name *Dahlgrenella farcta*, nov. gen. et nov. sp., is proposed for this form. The generic name *Dahlgrenella* is suggested to express my indebtedness to Professor Ulric Dahlgren of Princeton University for his kind assistance and advice in this work, as well as for his method of collecting mud-living hydroids.[1] The specific name *farcta* is descriptive of the method of asexual reproduction described above, being the Latin word for "stuffed." The ovoid bodies resemble a string of sausages, the word for which in Latin is "farcimen" from the same root as *farcta.*

Doctor Willie W. Smith of New York University was particularly helpful in the preparation of the manuscript for publication. I also wish to express my thanks for the facilities given me at the Mount Desert Island Biological Laboratory.

Genus Dahlgrenella

Characteristics: Hydroid stage (1) solitary.

(2) single whorl of oral tentacles.

(3) whorl of basal tentacles ringed with nematocysts.

(4) clusters of medusæ buds just oral to basal tentacles.

(5) asexual reproductive bodies found in perisarc posterior to foot.

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[1] Netting is slowly dragged over the bottom. A piece 2 by 4 feet is a convenient size with a one-half inch mesh. The forward edge is fastened along a board 2 feet by 2 inches to which is lashed a 2-foot length of one-inch copper pipe.

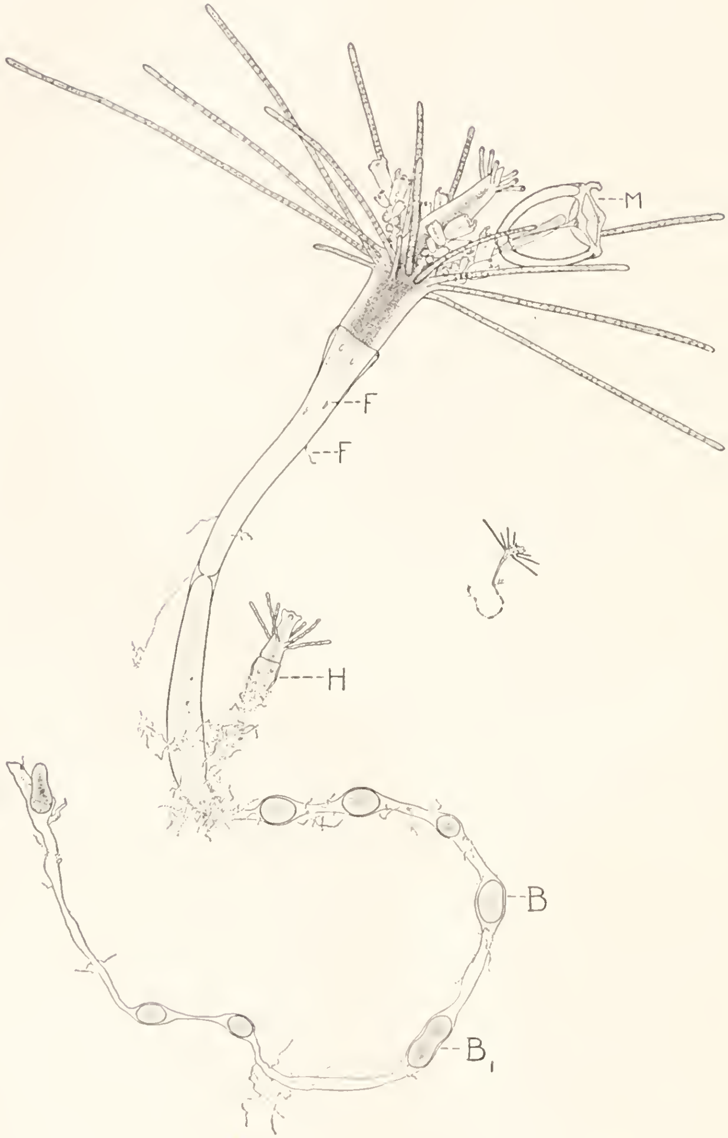

PLATE 1. *Dahlgrenella farcta*, mature hydroid with medusæ, *M*, and reproductive bodies, *B*. Reproductive body dividing, B_1. Young hydroid, *H*, probably produced by reproductive body and adhering to hydrorhiza. Frustules, *F*.

Medusa stage (only young medusæ observed)	(1) single short tentacle.
	(2) tentacle bulbs with red pigment.
	(3) nematocysts scattered over bell.

Size: Hydroid stage—about 5 mm. exclusive of hydrorhiza.
Medusa stage—young individuals about 1 mm.

Found: Mount Desert Island, Maine; on mud bottom between 40 and 60 feet.

Genotype: at the Smithsonian Institute of the National Museum, Washington, D. C.

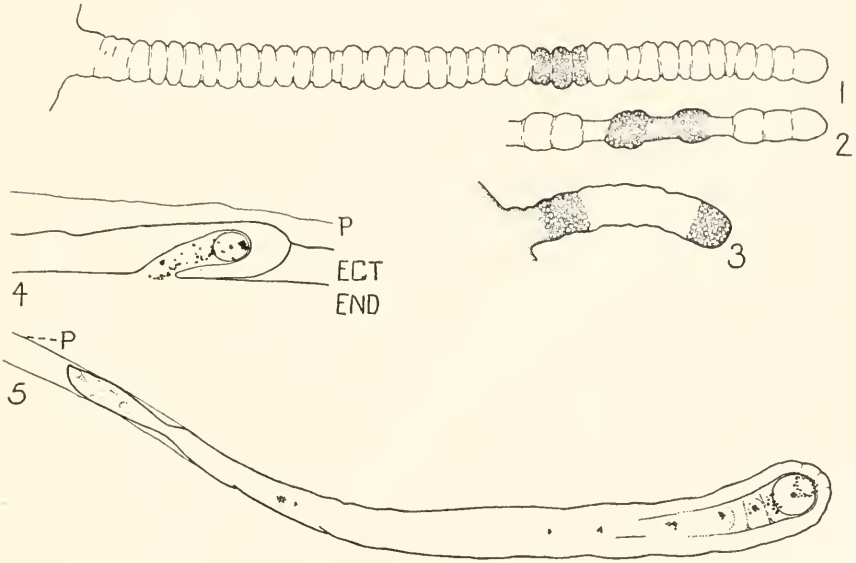

Plate II. *Dahlgrenella farcta,* basal tentacle, contracted, 1; basal tentacle, expanded, 2; oral tentacle, 3; young frustule, 4; fully developed frustule, 5 (perisarc, *P;* ectoderm, *ECT;* entoderm, *END*).

The hydranth is approximately 5 mm. long exclusive of hydrorhiza which may extend 15 mm. or more. There is a ring of from 6 to 8 capitate oral tentacles around the hypostome as well as a whorl of from 8 to 16 filiform basal tentacles (Plate II). The nematocysts are scattered evenly over the former while they are arranged in rings along the whole length of the latter. The body of the hydranth is also evenly covered with nematocysts. The hydranth extends aborally as a sort of foot secreting the perisarc. Posteriorly the perisarc forms a sticky hydrorhiza enveloping a string of ovoid bodies. The hydranth is pinkish or creamy white in color and an opaque white area is present within the ring of oral tentacles.

Frustules somewhat similar to those of *Corymorpha* can be seen on the foot and posterior region of the hydranth (Plates I and II). These develop into tentacle-like processes of adhesive nature much thinner than the oral and basal tentacles and covered with perisarc. The tip may adhere to some surface such as the bottom of a glass container and the cells connecting it with the foot will degenerate leaving only the strand of perisarc as anchorage. The young frustules just below the anterior edge of the perisarc are short protrusions while the more posterior frustules form the long anchorage fibers which may extend 3 mm. or more from the foot.

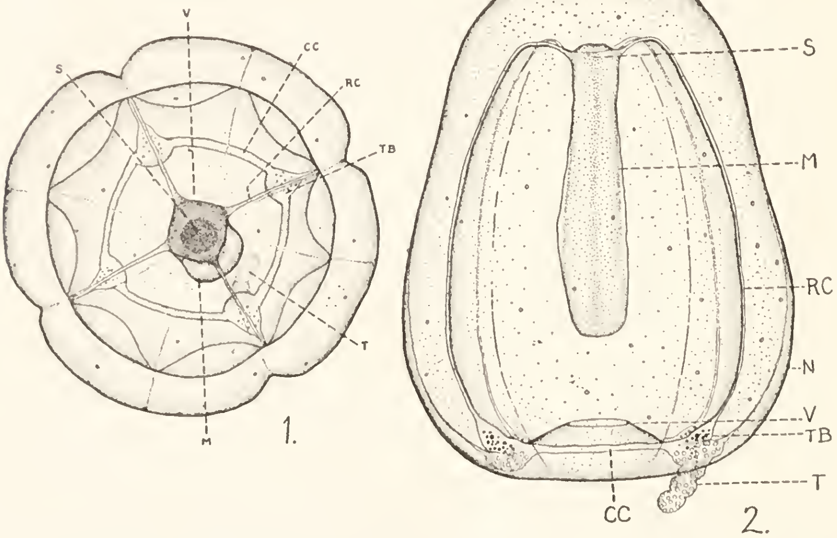

PLATE III. *Dahlgrenella farcta*, aboral view of slightly shrunken young medusa, 1; side view of normal young medusa, 2; circular canal *CC;* radial canal, *RC;* manubrium, *M;* stomach, *S;* single tentacle, *T;* tentacle bulb, *TB;* velum, *V;* nematocyst, *N.*

It is convenient to keep the living specimens for the examination of frustules and reproductive bodies on 75 by 50 mm. glass slides in stacking finger-bowls. The frustules will anchor the hydranth to the slide overnight so that they may be taken from one finger-bowl to another for changing the water and may be placed in Petri dishes for examining under the microscope without greatly disturbing them. By this means the attachment and movements of the more elongate frustules may be studied.

Clusters of medusæ (Plate III) and medusæ buds are located above the ring of basal tentacles. The young medusæ are about one mm. long when first set free. A single short tentacle densely covered with nematocysts is present. The four tentacle bulbs around the rim of the ovoid bell contain red pigment. In addition to those of the tentacle bulbs and tentacle, nematocysts are sparsely scattered over the entire bell.

The ovoid bodies (Plate IV) enveloped in the hydrorhiza are formed by fission of the foot and in some cases redivision of the parts constricted off from it. Rudimentary tentacles and frustules appear in

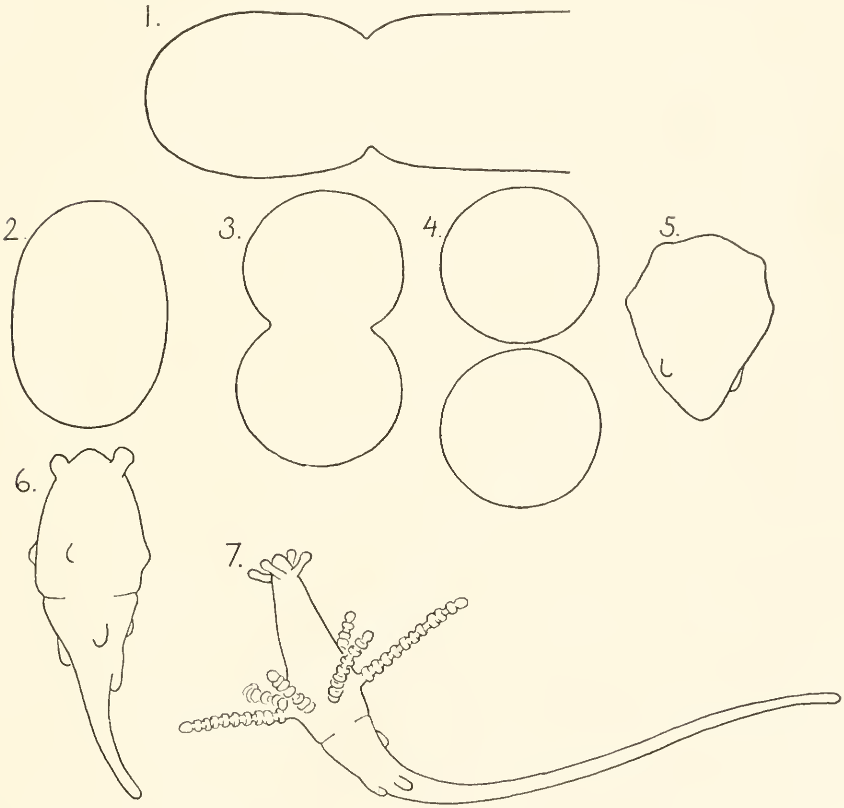

PLATE IV. *Dahlgrenella farcta*, development of asexual reproductive bodies (orientation not shown). Body constricting off from foot, 1; body after constriction (which may be followed by subsequent division), 2; body dividing, 3; body after division (which may be followed by further division), 4; developing hydroid with first appearance of tentacles and frustules, 5; older hydroid with appearance of perisarcal region, 6; older hydroid with well differentiated tentacles, 7. The hydroid usually penetrates the enveloping hydrorhiza between the stages 5 and 6.

the most posterior bodies first and development proceeds until the young hydroid is formed. As many as twenty bodies, each destined to develop into a new individual, have been observed in a single hydrorhiza.

It may be of some significance in the interpretation of this mode of reproduction that the young individuals appear to develop oriented oppositely from the parent. The hypostomata of the young in most cases point posteriorly in the hydrorhiza. Since two individuals pointed in the same direction may develop from a single body constricting equatorially, it is evident that the orientation is not dependent upon the location of the constriction. The possibility of the presence of an axial gradient should be investigated. However, the bodies become motile early and possibly reverse their positions by their movements. This possibility seems remote since one can observe in the position and structure of the sheath no conditions which might cause the bodies to point uniformly in a direction in which they had not developed.

It is extremely difficult to determine the orientation of the bodies for several reasons. First, the hydrorhiza is often rendered opaque by extraneous matter which has adhered to it. Second, it is impossible to distinguish oral from aboral end until frustules or tentacle buds appear. Third, soon after the appearance of frustules and tentacles the young hydroid penetrates the hydrorhiza thus allowing mechanical influences to disturb its orientation. Therefore this can be determined only under good conditions and only for a short period in the development of the bodies.

However, in a series of 17 specimens kept on glass slides over a period of 4 days, 25 bodies were observed which apparently pointed in the opposite direction from the parent while 2 seemed to point in the same direction. Of 7 other bodies whose orientation was more questionable 6 seemed opposite from the parent and 1 the same. In only 1 case was there evidence that the body had reversed its position. At the end of the 4 days in the 17 specimens there were 133 bodies of which 13 appeared during the last night.

The taxonomy of this form is fairly evident. The arrangement of tentacles in oral and basal whorls with medusa clusters located just above the former clearly indicates its relationship to the genera of the family Corymorphidae. It differs from *Corymorpha* and *Hybocodon* in having but one whorl of oral tentacles. This characteristic it shares with *Ectopleura*. The presence of a single marginal tentacle in the medusa is likewise indicative of its place with the *Corymorphidæ*. In this it is similar to *Hybocodon* and *Corymorpha* but differs from *Ectopleura*. The arrangement of tentacles as well as the distribution of nematocysts upon them is very similar to *Trichorhiza brunnea* which is generally classified in the family Pennariidæ as well as to *Heterostephanus annulicornis*.

The formation of a new genus for this hydroid seems justified not only because of oral tentacles, size, and form of hydrorhiza but especially because of the unusual method of asexual reproduction. An attempt to identify the medusa stage with any previously described form was also fruitless.

THE RELATION BETWEEN FOOD, THE RATE OF LOCOMOTION AND REPRODUCTION IN THE MARINE AMŒBA, FLABELLULA MIRA[1]

DWIGHT LUCIAN HOPKINS

(Department of Zoölogy, Duke University, and the Bermuda Biological Station for Research)

INTRODUCTION

It is known that the rate of locomotion in amœbæ varies with many of the factors in the environment, such as the osmotic pressure (Hopkins, 1928), the hydrogen ion concentration (Pantin, 1923; Hopkins, 1928; Mast and Prosser, 1932; Pitts and Mast, 1933 and 1934), the concentration of salts (Pantin, 1926*a* and *b*; Hopkins, 1929; and Pitts and Mast, 1933 and 1934), the nature of the substratum (Hopkins, 1929), and temperature (Schwitalla, 1924 and 1925; Pantin, 1924; Mast and Prosser, 1932, and Hopkins, 1937). It has been shown, however, that within wide limits the rate of locomotion is not affected by the oxygen tension (Hulpieu, 1930; Pantin, 1930*a* and *b*).

There have been no studies made of the relation of the rate of locomotion to the amount, concentration, or quality of the food which the amœba has had available. Does the ingestion of an abundance of food result in a high rate of locomotion, and conversely, does a scarcity of food result in a low rate of locomotion? Does the nature or quality of the food modify the rate of locomotion? In studies on the rate of locomotion, efforts have been made to maintain the food constant, but a careful consideration of these efforts reveals the fact that they are inadequate if the rate of locomotion is directly dependent upon the food supply. Surprisingly, however, it is observed that regardless of the lack of precise control of the food supply fairly consistent results have been obtained. Could it then be that the rate of locomotion is, within limits, independent of the amount, concentrations and quality of the available food?

The study to be reported here was an attempt to answer some of these questions and to compare this with the effect of food upon growth and reproduction. A marine amœba, *Flabellula mira* Schaeffer was used.

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[1] Contribution from the Bermuda Biological Station for Research.

MATERIAL AND METHODS

The amœbæ used in these experiments were from the same clone of *Flabellula mira* Schaeffer, used in recent work on the effect of temperature (Hopkins, 1937). Rice (1935), in a study of the nutrition of this same clone of *Flabellula mira*, found that they could live and reproduce normally and indefinitely on each of several pure strains of bacteria. Consequently I have used, in these experiments, pure strains of bacteria each of which alone would furnish sufficient nutriment for the amœba to live and reproduce normally and indefinitely. Bacteria elaborating substances toxic to the amœbæ were avoided. Six different bacteria were used. They were not identified but isolated, and the fact that they were distinctly different bacteria, established for me by Mr. Jump of the Botany Department of the University of Pennsylvania. They will be designated, Bacterium 1, 2, 4, 5, 7 and 8 respectively.

The experiments consisted of (1) a study of the growth and the rate of locomotion of amœbæ which had been fed bacteria from the pure strains No. 4 in different concentrations, (2) a study of the rate of locomotion of amœbæ fed on two concentrations of each of six bacteria, numbers 1, 2, 4, 5, 7 and 8 respectively, and (3) an experiment on the rate of locomotion in the absence of food particles.

The bacteria were cultured at room temperature in 1 per cent dextrose, 1 per cent Bacto-peptone in distilled water. Each bacterium was inoculated into 100 cc. of this medium, set aside and allowed three days for incubation. At the end of this period the bacteria were centrifuged from the culture medium and washed three times with distilled water. Finally the washed bacteria were diluted with artificial sea water (Butts, 1935) to 100 cc. This stock suspension of bacteria was then diluted as desired with artificial sea water. An equal quantity of each dilution of each bacterium in a Petri dish was inoculated with a definite quantity of culture fluid from a well stirred amœba culture. The same amount of culture fluid was used to inoculate each of the several cultures of a given experiment. One inoculating culture only was used for inoculating all of the cultures of the first set and another for the second set of experiments.

It should be pointed out that by washing the bacteria and finally placing them in artificial sea water, practically all nutrient material for the bacteria was eliminated. Thus growth and multiplication of the bacteria during the experiment was maintained at a minimum.

Method of Measuring the Growth of the Amœbæ in the Cultures

Since the number of amœbæ with which the different experimental cultures were inoculated was approximately the same for each culture, the concentration of amœbæ in each culture after a given growth period would give a value which would be an index of the relative growth in the culture, i.e., in comparison with the other cultures of the same series. The concentration of amœbæ was obtained by counting the number of amœbæ occurring on a square millimeter of the surface film, plus the number occurring on a square millimeter of the bottom of the dish. In an undisturbed culture all of these amœbæ occur either on the bottom of the dish or on the surface film. In order to obtain a representative value for each culture the number of amœbæ in each of ten surface-films plus bottom-millimeter squares were counted and averaged.

Rate of Locomotion

The rate of locomotion was obtained as described in a recent paper (Hopkins, 1937) except that in those experiments I was able to equalize the error due to the presence of bacteria in the locomotion medium by inoculating equal quantities of culture fluid containing amœbæ and bacteria from the same culture into equal quantities of the locomotion medium. But since the amœbæ in the present experiments must come from cultures containing different concentrations of bacteria, it was not possible thus to equalize this error.

By recording the rate of locomotion only when this amœba has a large clear and smooth hyaline edge, most of the error due to the presence of bacteria or other food particles is eliminated. In feeding, this amœba forms food cups mostly in the hyaline edge. When a food organism is contacted by the hyaline edge, a cup is formed at the point of contact. The protoplasm of the hyaline edge concentrates in the region of the cup and diminishes considerably the area of the hyaline edge. Then the cup with the captured food is drawn into the body of the cell. This process can be detected under low power mainly by the decrease in area of the hyaline edge. Oftentimes, however, the feeding process passes unobserved, resulting in a negative error in the observed rate of locomotion.

By washing the amœbæ several times the bacteria may be eliminated. But this procedure is very tedious due to the small size of the amœbæ, and required for these experiments more time than was available, since it was necessary to measure the rate of locomotion of the amœbæ in all the cultures of a series in as short a time as possible. In view of these considerations, the expedient used to equalize the error was to dilute the drop of culture fluid containing the amœbæ with bacteria-free artificial sea water according to the concentration of bacteria known to be in the culture, thus approximately equalizing the concentration of bacteria in the locomotion medium at the time when the rate was observed.

RESULTS

The Relation between the Concentration of Food, Growth, and the Rate of Locomotion in Flabellula mira

A stock suspension of Bacterium 4 was diluted 2, 4, 8 and 10 times with artificial sea water and two portions (20 cc. each in a Petri dish) of each dilution inoculated with equal numbers of amœbæ. In addition two cultures were set up by merely adding the same quantity of inoculating fluid and amœbæ to 20 cc. of artificial sea water. All of the cultures were allowed to develop at room temperature (18° to 23° C.).

TABLE I

Showing the relation between the rate of locomotion and the concentration of Bacterium 4 available as food. X is concentration of bacteria in the stock suspension, X/2, X/4, X/8 and X/10 various dilutions. Concentration y indicates the concentrations of bacteria of cultures to which only the bacteria with the inoculating amœbæ were added. Rate in micra/minutes in artificial sea water. Temperature 21.6° + 0.2° C.

Concentration of Bacterium 4	$X/2$	$X/4$	$X/8$	$X/10$	y
Third day		27.0	27.5	28.0	29.5
Fifth day	26.0	30.0	27.0	29.0	29.0
Eighth day	30.0	31.0	33.0	30.0	33.0
Average	28.0	29.3	29.2	29.0	30.5
Average for all experiments	29.2				

On the third, fifth and eighth days samples of amœbæ were removed from each culture and diluted with artificial sea water, as described above, and the rate of locomotion determined for five minutes for each of the five amœbæ at 21.6 ± 0.2° C. The results for each culture were averaged and this average was averaged with the other culture of the same dilution. The results are presented in Table I.

On the seventh day the concentration of amœbæ in each culture was observed and the results for each culture averaged with results from the other culture of the same dilution. The results are presented in Table II.

From the results presented in Table I it is quite clearly shown that within the limits here studied the concentration of bacteria which has been available to the amœbæ as food has no influence on the rate of locomotion.

From the results presented in Table II it is shown that the growth of these amœbæ increases as the concentration of the bacteria increases. The highest concentration of bacteria used here gave the greatest, and the lowest concentration the least amount of growth.

The Relation between the Kind and the Concentration of Bacteria Available as Food and the Rate of Locomotion

In these experiments the amœbæ were fed on two concentrations of each of the six bacteria 1, 2, 4, 5, 7 and 8. The amœbæ were inoculated into 20 cc. of the stock suspension of each bacterium prepared as described above and into 10 cc. of the stock suspension diluted to 20 cc. with artificial sea water. The rate of locomotion, in artificial sea water at 21.6 ± 0.2° C., of amœbæ fed for five days on each concentration of bacteria, was obtained by averaging the results of five minutes locomotion for each of five amœbæ. The results are presented in Table III.

Table II

Showing the relative growth of amœbæ fed on different concentrations of bacteria for seven days. The concentrations of the bacteria are the same as in Table I. Temperature the same for all cultures. Amœbæ per sq.mm. of bottom + sq.mm. of surface film.

Concentrations of bacteria	$X/2$	$X/4$	$X/8$	$X/10$	y
Culture I	25.0	9.2	9.0	10.0	8.2
Culture II	18.0	28.8	12.6	7.9	5.6
Average	21.5	19.0	10.8	8.9	6.9

From the results of Table III it is observed that the rate of locomotion of these amœbæ has not been influenced by the variations in kind and concentration of the bacteria on which they have been fed.

The Rate of Locomotion of Flabellula mira in Artificial Sea Water at 21.6 ± 0.2° C. and its Constancy When All Food Particles are Absent from the Medium

By a food particle is meant here any particle which the amœba will ingest. Since, as has been shown, the rate of locomotion of *Flabellula mira* is within wide limits independent of the amount and kind of food it has had available, it seemed worth while to determine accurately the rate under definite and reproduceable conditions. Also the question arose as to whether the rate of locomotion in the absence of external stimulation is variable or constant. Schwitalla (1924) has maintained that locomotion in *Amœba proteus* is never constant but varies in a rhythmical fashion. Hahnert (1932) and Pitts (1933), however, have shown that Schwitalla's rhythms were due to the fact that in measuring the rate he did not take into consideration the changes in form of the amœbæ while observing the rate. They maintain that in *Amœba proteus* when the form is monopodal the rate of locomotion is constant.

In the experiment now to be described, the object was to remove from the environment of the amœbæ, as much as possible, all chances of stimulation from the environment. The main and most frequent stimulation from the exterior to which they react is to particles which they take in as food. They engulf not only bacteria but in addition yeast, carbon particles, dust, etc. In order to eliminate interference to locomotion due to such particles the following procedure was followed.

Table III

Showing the relation between the kind and concentration of bacteria and the rate of locomotion. X is concentration of bacteria in the stock suspension, and X/2 the dilution. Rate in micra/minutes in artificial sea water. Temperature 21.6 ± 0.2° C.

Bacterium No.	1	2	4	5	7	8
X	28.0	27.0	30.0	28.0	32.0	30.0
X/2	32.0	28.0	30.0	26.0	29.0	28.0
Average	30.0	27.5	30.0	27.0	30.5	29.0

Average for all experiments 29.0

The Pyrex glass dishes, or locomotion dishes, were washed in sulfuric acid-potassium bichromate cleaning solution, rinsed several times in distilled water, dried, polished with a clean cloth and placed on a sheet of platinum and heated to a red heat for about ten minutes. In this way the surface of the dish was freed of all organic particles. The artificial sea water was filtered through three layers of No. 50 Whatman filter paper, after the filter paper had been washed thoroughly by running through it several hundred cubic centimeters of artificial sea water. The amœbæ were washed free of bacteria by removing them singly from the culture with a sterile capillary pipette and passing them through five changes of the filtered artificial sea water. They were finally placed in filtered artificial sea water in the cleansed Pyrex glass dish. The dish was then covered with a thoroughly cleansed cover-slip to keep out dust from the air. The dish containing the amœbæ was placed in the observation chamber of the constant temperature apparatus

(Hopkins, 1937) at 21.6 ± 0.2° C. Cultural temperature for the amœbæ used was from 18° to 22° C. Thus no adaptation to temperature (Hopkins, 1937) was necessary before locomotion could become constant. One hour, however, was allowed for the amœbæ to recover from the effects of handling and the small change in light intensity associated with the artificial light used for the observations. The environment thus established was remarkably free of external stimulating agents. The medium, however, still contained a few bacteria and small particles which could be seen under low-power of the microscope. These were very infrequent such that they could be detected before the amœbæ under observation contacted them. The performance of each of three amœbæ was observed. The observation on each amœba was continued until it contacted and engulfed a visible particle which resulted in a temporary cessation of locomotion. During the periods of observation these amœbæ contacted a few particles which they did not engulf, and no interruption in the locomotion occurred. Amœba A traveled 55 minutes before it engulfed a particle and ceased temporarily to move; B 18 minutes, and C 25 minutes. The minute to minute performance of each of these three amœbæ is given in Table IV. In the table the distance traveled each minute was measured to the nearest magnified millimeter.

TABLE IV

Showing the performance of three amœbæ in the absence of food particles. Temperature 21.6° C.

Amœba	Distance in mm. traveled by amœbæ in each of a number of consecutive minutes × 118	Rate micra/min.
A	4, 4, 3, 4, 3, 4, 4, 4, 4, 4, 4, 3, 4, 4, 4, 5, 5, 4, 4, 4, 4, 4, 4, 4, 5, 3, 4, 4, 4, 4, 4, 4, 4, 5, 4, 4, 4, 4, 4, 4, 4, 4, 3, 4, 5, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4.	33.9
B	3, 4, 4, 5, 4, 4, 4, 4, 4, 3, 3, 3, 3, 3, 4, 4, 4, 5.	31.9
C	4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4, 4.	33.9
Average		33.23

The results show that no rhythms occur in the rate of locomotion of this amœba and that as long as it received no stimuli from the environment, its rate of locomotion is constant.

When we average the rates obtained in the preceding experiments, a value of 29.1 micra/min. is obtained for the rate of locomotion of these amœbæ in artificial sea water at 21.6 ± 0.2° C. and in the presence of a relatively few food particles. This is 4.13 micra/min. low when compared with the rate obtained in the last experiment, a negative error of aproximately 13 per cent. This error is undoubtedly due to the presence of food particles in the environment, and appears to be unavoidable if food particles are present in any abundance. The interference to locomotion due to the presence of food probably occurs only after the amœba has come in contact with the food since Rice (1935) has shown that this amœba cannot sense its food at any distance. After ingestion of food it is possible that the amœba does not immediately resume its normal rate but accelerates slowly.

Discussion

These results appear to be very definite and far-reaching in their implications. It must be pointed out, however, that they cannot be taken to mean that food has no influence upon the rate of locomotion. Food, of course, must be available to furnish the energy necessary for locomotion. The concentrations of food studied in these experiments do not include extremely high or low concentrations. It is very probable that in these amœbæ when the food becomes scarce and the energy necessary for locomotion is no longer available, the rate of locomotion is lowered. It is also possible that when the food organisms become too plentiful the excretion products become sufficiently concentrated to affect the rate of locomotion. It was felt, when these experiments were planned, that the higher concentrations used were sufficient to produce an effect on the rate of locomotion. That this was not the case has been seen. Repeated efforts have been made to actually starve these amœbæ, but the technique involved is very difficult and a satisfactory method has not yet been devised. It may be that starvation to such an extent that the energy necessary for locomotion is not available, results in a rapid decrease in the rate of locomotion to zero, followed immediately by encystment, or that encystment may take place before the available energy has fallen to such a low level.

It is quite evident, however, that when an excess of food is ingested by this amœba it is not utilized by an increase in the rate of locomotion but is utilized by an increase in the rate of growth and reproduction. This procedure, of course, has a distinct survival value. On the other hand, the fact that when food becomes relatively scarce the rate of locomotion is not decreased has a survival value in that the amœbæ are better able to reach regions where food is more abundant. Failing of this the most expedient reaction, viewing the matter in the light of possible survival, would be to encyst and remain thus until food in the environment is again plentiful. It has been shown (Beers, 1926) that a deficiency of food results in encystment in *Didinium*.

With reference to the possibility that these findings for the relation of the rate of locomotion to food in *Flabellula mira* hold for the other amœbæ, it might be said here that experiments now in progress indicate that they hold for fan-shaped amœbæ but not for limacine amœbæ.

Conclusions

1. The rate of locomotion of *Flabellula mira,* within limits, is independent of the amount of food it has had available.

2. Growth and reproduction of *Flabellula mira* are dependent on the concentration of the food available.

3. The rate of locomotion of *Flabellula mira* is independent of the kind of bacteria available as food, provided the bacteria are adequate for normal growth and reproduction.

4. In the absence of external stimuli and in a normal environment to which it is completely adapted, the rate of locomotion in *Flabellula mira* is constant, as far as can be observed under a magnification of 118 diameters, and is very close to 33 micra per minute at 21.6° C.

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THE CHROMATOPHOROTROPIC HORMONE OF THE CRUSTACEA: STANDARDIZATION, PROPERTIES AND PHYSIOLOGY OF THE EYE-STALK GLANDS[1]

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STANDARDIZATION

The need for a method of determining quantitatively the amount of hormone in a given extract of a gland is obvious in chemical or quantitative physiological work dealing with endocrine systems. Although the existence of such a system for the pigmentary effectors of crustaceans has been known for almost ten years, no reliable method for the standardization of the eye-stalk hormone has been devised. Navez and Kropp (1934), and Kropp and Crozier (1934) suggested that the eye-stalk hormone may be standardized by the *Avena* coleoptile method since they found that the eye-stalk extract accelerates the growth rate of *Avena* coleoptiles. These authors employed water extracts of the eye-stalks of *Palæmonetes.* Since many other substances in addition to the chromatophore hormone are present in a water extract of the eye-stalks, it is not certain that the growth effects on plants are due actually to the chromatophore hormone. On the basis of biochemical evidence, Carlson (1936) suggested that the growth reactions of plants to the eye-stalk extract was due to the presence of some substance other than the chromatophore hormone. If this is true, it would then appear that Kropp and Crozier have not been measuring the chromatophore hormone but possibly some other substance present in a water extract of the eye-stalks.

Consequently, it was felt that the most reliable way of assaying the chromatophore hormone would be to measure the response of the tissue (the chromatophores) which this hormone normally affects.[2] A convenient laboratory animal on which a method of assay may be devised is the fiddler crab, *Uca.* In the vicinity of Woods Hole, two species, *U. pugilator* and *U. pugnax,* are abundantly present. Both these spe-

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[1] Aided in part by a grant from the Rockefeller Foundation, administered by F. L. Hisaw.

[2] Cf. Burn, J. H., 1930. *Phys. Rev.*, **10**: 146.

cies contain black, red, white and yellow integumentary chromatophores. Only occasionally, however, are red chromatophores observed in *U. pugnax*. The extreme color change of *U. pugnax* ranges from jet black to pale yellow (Figs. 3, 4) and of *U. pugilator* from dark brown to cream white (Figs. 5, 6). The melanophores are the chief agents of this change although the movements of the other pigments are instrumental in producing the end result. Megašur (1912) demonstrated that extirpation of the eye-stalks of *Uca* resulted in the production of the pale phase of its color change due to melanophore contraction. Carlson (1935, 1936) confirmed this observation and interpreted it as an effect specifically due to the loss of the endocrine glands contained in the eye-stalks. An extract of the extirpated eye-stalks when injected into a pale (blinded) animal produced the dark phase resulting from melanophore expansion. Carlson showed further that if 0.1 cc. of a solution containing the extract of 1 eye-stalk of *Uca* in 1 cc. of water was injected into a blinded[3] crab, the melanophores became expanded within 2 hours, remained expanded for about 4 hours, and finally contracted so that the animal resumed the pallor previous to injection. This is specifically a hormonal reaction. There appears to be no other way whereby the melanophores of a blinded *Uca* may be induced to expand.

Preliminary Experiments

The statements of Megašur and Carlson concerning the effects of blinding and of injection of eye-stalk extracts were first confirmed (Figs. 3, 4, 5, 6). The typical response of a blinded animal following the injection of an extract of its eye-stalks in a dose equivalent to 1/20 of a single stalk may be conveniently divided into four phases:

(*a*) The first perceptible response.

This response is evidenced by the beginning of melanophore expansion and occurs from 15–20 minutes after the injection has been made.

(*b*) The attainment of the maximal effect.

After the first perceptible response has occurred, the melanophores become maximally expanded within one hour.

(*c*) Duration of melanophore expansion.

Following the attainment of the maximal effect, the melanophores remain fully expanded for about 3¼ hours.

(*d*) The period during which the melanophores contract again.

Four and one-half hours following the time of injection, the

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[3] The term *blinded* will be used throughout this paper to designate the condition of specimens whose eye-stalks have been completely removed.

> melanophores begin to contract, and within the following half-hour, the animal becomes again pale, so that the melanophores are in the same condition as that previous to the injection.

Preliminary experiments were therefore designed to determine the relation between each of the four phases of the response and concentration of hormone. A sea water extract was prepared from 280 eye-stalks of *Uca pugnax* and a series of the ten following dilutions was made: 8, 4, 2, 1, 0.5, 0.25, 0.12, 0.06, 0.03, 0.015 E.S.[4] per cc. of solution. Several hundred specimens of *Uca pugnax*, blinded two days previously, were employed as test animals. All animals were of equal size and weight (3.5 grams). The injection volume in all experiments was 0.1 cc. Fifty animals in groups of 5 constituted the experimental material for each test. The tests were carried out by injecting each group of 5 animals with but one of the 10 dilutions so that each animal in the first group received a dose of 0.8 E.S. in the second group 0.4 E.S., in the third 0.2 E.S., etc. It was found that the time at which the first perceptible response occurred was independent of the concentration of hormone. In all ten groups the first signs of melanophore expansion occurred 15–20 minutes following the time of injection, although at the two lowest concentrations (0.0015 E.S. and 0.003 E.S.) this time was slightly increased. The attainment of the maximal effect was likewise found to be independent of concentration when concentrations higher than 0.003 E.S. were used. Concentrations below this value did not produce the maximal response. The duration of melanophore expansion, however, was found to be proportional exponentially to the concentration of hormone employed (doses above 0.003 E.S.). The time during which the melanophores became contracted again was, like the first two phases of the response, independent of the concentration.

The significant point of these experiments is the demonstration that the duration of melanophore expansion varies as a function of concentration. The times for the completion of the other three phases of the response are for all practical purposes constants, independent of concentration provided the doses employed are greater than 0.003 E.S. In order to measure the response of the animals to a given concentration, it is therefore necessary to determine only the time of injection and the time at which the animals become again pale. The difference in these two readings is obviously the duration of the response plus 1.75 hours, which may be used as a constant equal to the sum of the times of the other three phases of the response.

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[4] The letters E.S. will be used throughout this paper as an abbreviation for the extract equivalent to a given number of eye-stalks. For example, 1 E.S. indicates that the extract is equivalent to that obtainable from one eye-stalk; 0.5 E.S. denotes an extract equivalent to half that obtained from one eye-stalk, etc.

Variables in the Method of Assay

There are several important variables which must be controlled completely if such a method of assay is to be used.

(1) *Physiological Uniformity of the Responsive Tissue.*—The black chromatophores were used as the sole criterion in obtaining readings since they are the chief instruments in determining the appearance of the animal with respect to paleness or darkness. In blinded specimens of *Uca* the pigment in all melanophores of the appendages and body is uniformly and extremely concentrated, and remains so indefinitely regardless of environmental conditions which would provoke expansion in a normal animal. Thus it appears certain that the responsive tissue in all test animals is in a physiologically uniform state.

TABLE I

Size of eye-stalk in relation to hormone concentration

Size of Eye-stalk	Injected Dose	No. Tested	Average Weight of Test Animals	Average Response
			grams	*hours*
Small (animal weight, 1.7 grams)	0.025 E.S./cc.	8	3.5	3.75
Medium (animal weight, 2.8 grams)	0.025 E.S./cc.	8	3.6	4.09
Large (animal weight, 5.1 grams)	0.025 E.S./cc.	8	3.5	4.88

(2) *Concentration of the Injected Hormone.*—This variable is easily controlled since the method of preparing the extract unless otherwise stated was maintained constant. The eye-stalks were cut off and ground in a small mortar in the amount of sea water necessary for any particular concentration. This extract was brought to a boil so that a coagulum (presumably the tissue proteins) formed. The solution was filtered, and the filtrate made up by the addition of unboiled sea water to the desired concentration. About 0.6 cc. of the sea water was usually lost during the process of boiling and filtration. This resulted in a slightly hypertonic extract, but this factor is immaterial for the purpose of the experiment (cf. Carlson, 1936). The solution was allowed to cool to room temperature, usually about one-half hour elapsing, and the injection made. While the concentration of the hormone in terms of numbers of eye-stalks can be controlled quite accurately, the size of the eye-stalks used in making an extract of a certain concentration could not be controlled so easily. Larger eye-stalks may contain large glands and hence more hormone, as shown by Table I. To control this factor,

the extracts were prepared from eye-stalks of animals of a definite and uniform size.

(3) *Size of the Test Animal.*—This variable proved to be a very disconcerting factor at the beginning of the work. It was reasonable to suppose that large animals would show a smaller response to a given dosage than small animals because they would contain a greater amount of the responsive tissue, and because the hormone would be subjected to greater dilution. That this is plausible is shown by Table II.

Consequently, in order to avoid the use of a curve for the calibration of response as a function of the size of the test animals, assay experiments were always carried out on animals of uniform weight.

(4) *Individual Variation.*—This variable is probably the most significant of those already mentioned because it is not easily controlled. Since it is almost impossible to carry out all experiments on the same animal, the best method would be to use sufficient numbers of animals and to treat the data statistically. In performing an assay, from 15 to 20 animals of the same size and weight were injected with the same volume and concentration of hormone and the average response taken. The standard deviation of the arithmetic mean was calculated and determinations made to see if the differences in the response at different concentrations were really significant.

TABLE II

Size of test animal in relation to response

Hormone Concentration	No. of Animals Tested	Average Weight	Average Response
		grams	*hours*
1.0 E.S./cc.	10	4.36	4.13
1.0 E.S./cc.	6	3.86	5.56
1.0 E.S./cc.	10	1.55	7.83

(5) *Volume of Injected Dosage: Place of Injection.*—Several experiments made to determine the most suitable volume of hormone to be injected led me to adopt finally 0.05 cc. as the standard injection volume. Carlson used 0.1 cc. but while this is satisfactory for *U. pugnax* and very large specimens of *U. pugilator*, it seemed to be too great a dose for the size of crabs employed in most of my experiments. The most convenient region for the injection of the hormone into the body spaces was found after many trials on different regions of the body to be through the soft tissue forming the joint between the coxipodite and the protopidite of the walking legs. This method allows for speed and

accuracy of injection for, using a 27-gauge hypodermic needle, one can inject 20 crabs within 2 minutes.

Method Finally Adopted

Upon the basis of these preliminary experiments, the standardization of the eye-stalk hormone was completed using blinded *Uca pugilator*[5] as a test animal. One hundred and fifty such specimens, whose eye-stalks were extirpated two days previously, were arranged in ten groups of 15 animals each. All animals were of uniform size and weight (2.46 ± .01 gram). A stock solution of hormone was made by extracting the extirpated eye-stalks as previously described. A series of 10 concentrations ranging from 5 E.S./cc. to 0.03 E.S./cc. was prepared. Five-hundredths of a cc. of these concentrations was used as the injection volume in all cases. Each of the 15 animals in a particular group received 0.05 cc. of a particular concentration, so that each of the ten groups of animals was injected with but one of the ten various known concentrations. The difference between the time of injection and time at which the animals became pale again was noted. The results of this experiment are shown by Fig. 1. The experiment was repeated five times, and although the points obtained did not fit the curve as well as those of Fig. 1 do, it was generally true that the magnitude of the response was exponentially proportional to concentration.

It can be seen from Fig. 1 that the relation between the complete response of the melanophores and the concentration of hormone is least sensitive over the range of concentrations above 1.0 E.S./cc. and most sensitive over the range from 0.06 E.S./cc. to 1.0 E.S./cc. This sensitive range is re-plotted in Fig. 2, which shows that there is a linear relationship between the duration of melanophore expansion and concentration of hormone.

Figure 2 was therefore used to establish the *Uca* unit. Two possibilities are open. One can choose an arbitrary response near the midpoint of this curve and designate this value as one *Uca* unit. Assays would therefore be made by repeated dilutions of an unknown until the resulting response equals that chosen to represent one *Uca* unit. A second possible method would be to inject 0.05 cc. of an unknown concentration, obtain the average response and read off from Fig. 1 the concentration in terms of *Uca* E.S./cc. which gives the same response. The latter procedure was adopted because both amount to the same thing provided the unknown concentration falls within the sensitive range.[6] The response (4.9 hours) produced by 0.05 cc. of an extract of 1 E.S. of *U. pugilator* (2.46 grams) per cc. of solution was designated as one *Uca* unit.

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[5] *Uca pugilator* was substituted for *U. pugnax* as the standard test animal because it is much more uniform in weight and size and because the rate of mortality and the frequency of autotomy are much lower than in *U. pugnax*.

HORMONE CONTENT IN THE EYE-STALKS OF VARIOUS CRUSTACEANS

In estimating the hormone content in the eye-stalks of various crustaceans, the following method was used: a number of eye-stalks were cut off, extracted with sea-water, boiled, filtered, and in all cases the solution was made up so that 1 cc. contained the extract equivalent

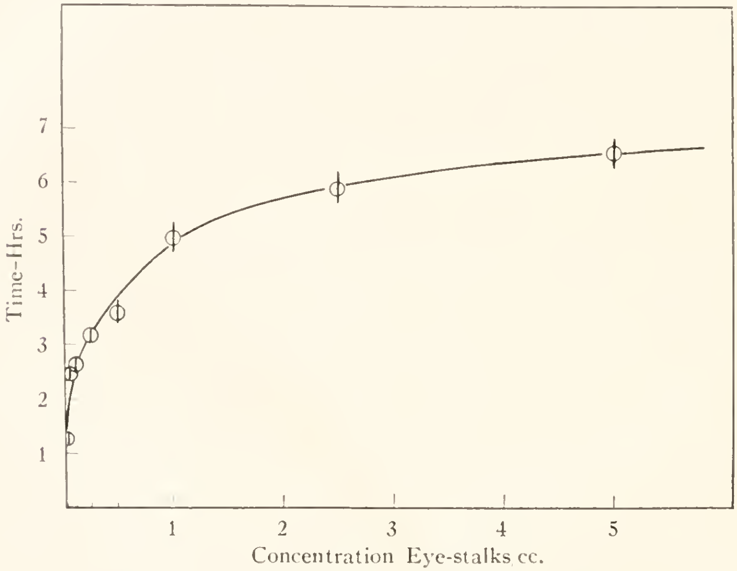

FIG. 1. Curve showing the relationship between the concentration of the hormone and the complete response of *Uca pugilator*. The points are averages of 15–20 animals, over 150 animals being used in this particular experiment. The solid bars represent values equal to twice the standard deviation of the arithmetic mean.

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[6] This range is sensitive enough to detect differences in response produced by a concentration of X, and one of 2X. The significant differences between some of the points along this range are much greater than that required by the standard equation $\frac{S.E._1 - S.E._2}{\sqrt{\sigma^2_1 + \sigma^2_2}} =$ or > 3. The sensitivity of the curve would be increased by obtaining more points along this range, and by using extremely large numbers of animals to cut down the size of the standard error.

to 1 eye-stalk.[7] Five-hundredths of a cc. of the solution was injected into each of 15 animals and the average of the responses taken. The concentration in terms of *U. pugilator* E.S./cc. which produces the same average response was read off from Fig. 1. The hormone content of one eye-stalk of various crustaceans as compared with one eye-stalk of *U. pugilator* (1 unit) is given in Table III. The extracts were always made during the day and from animals which had been maintained under illumination (daylight).

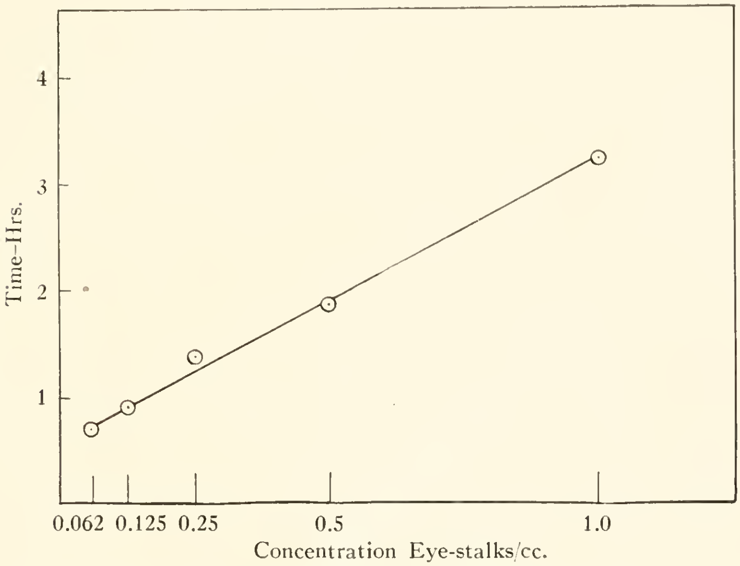

FIG. 2. Curve showing relationship between the duration of melanophore expansion and concentration of hormone (values replotted from Fig. 1). Theoretically, the curve should go through the 0 point.

EXTRACTION AND PURIFICATION

Although ten years have elapsed since the eye-stalk hormone was discovered, very few chemical properties of the hormone are known. Perkins showed that the hormone was soluble in water, and that it was resistant to boiling. Carlson (1936) and Abramowitz (1936*a, c*) demonstrated that it was soluble in alcohol but insoluble in ether. Carlson also added that the hormone was not destroyed when treated for short periods of time with acid or alkali.

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[7] Unfortunately this proved to be a mistake because a few readings obtained in assaying the potency of the eye-stalk extracts of certain crustaceans were not within the sensitive range, and therefore cannot be considered as accurate readings.

Since a method for standardizing the hormone has been devised, an attempt was made to purify the substance. One thousand eye-stalks of *Uca pugilator* were dried and pulverized. The total activity of the dry powder was 1000 *Uca* units, or 0.6 *Uca* units per mg. of dry powder. This material was extracted several times with small volumes of light petroleum ether in order to remove a red carotenoid pigment which was found to contaminate the subsequent fractions. The ether solution was washed with small amounts of distilled water. The water layer was then added to the residue insoluble in ether and the ether layer being only slightly active was discarded. The residue which was insoluble in ether was then extracted three times with distilled water. The water solution was boiled and filtered, and the filtrate dried in a current of warm air. The dried material was washed with small amounts of chloroform to remove traces of the red pigment, and the chloroform washings being only slightly active were discarded. The dried filtrate was then extracted with 95 per cent ethanol, and the alcohol solution was centrifuged. The alcohol-soluble fraction was decanted and dried. Both the alcohol-soluble and alcohol-insoluble fractions were active, the latter being much less so than the former, and hence was discarded. The material soluble in 95 per cent ethanol was then dissolved in hot absolute alcohol and precipitated by the addition of ether. The activity of the material soluble in absolute alcohol was approximately two *Uca* units per mg. Further attempts to precipitate the material proved unsatisfactory because of the exceedingly small yield. The loss in total

TABLE III

Hormone content of the eye-stalks of various crustaceans

Average Weight of Animals	Species	*Uca pugilator* Units
grams		
2.46	1 E.S. *Uca pugilator*	1.0
3.3	1 E.S. *Uca pugnax*	1.0
11.0	1 E.S. *Pagurus pollicaris*	1.25
309.0	1 E.S. *Homarus americanus*	1.20
2.0	1 Head *Hippa talpoida*	0.25
0.38	1 E.S. *Crago borealis*	0.25
219.0	1 E.S. *Carcinus mænas*	1.25
80.0	1 E.S. *Cancer irroratus*	5.0
0.4	1 E.S. *Palæmonetes vulgaris*	0.36
15.0	1 E.S. *Uca minax*	1.5
127.0	1 E.S. *Libinia dubia*	4.0

activity was about 60 per cent. The material which is soluble in absolute alcohol is devoid of pigment and is apparently protein-free.[8]

Properties of the Hormone

The eye-stalk hormone is readily soluble in water, but not completely soluble in ethanol or methanol. It is only slightly soluble in acetone, and insoluble in organic solvents such as benzin, chloroform, or ether. The hormone is thermostable, but is destroyed by oxidation. It does not decompose when boiled with HCl or NaOH in a 1 per cent solution for short periods of time. If the hormone is boiled for 2 hours with NaOH, the activity is completely destroyed. The hormone adsorbs easily to various substances present in crude extracts of the eye-stalks. If the eye-stalks are extracted with benzin, and the benzin-soluble material (chiefly pigment) and the benzin-insoluble material tested in equivalent doses, only the benzin-insoluble is found to be active. However, if the benzin-soluble material is concentrated and then tested, some activity will be found. The same phenomenon was observed when working up sea-water extracts of the eye-stalks of various crustaceans. These extracts were dried, and the dried material extracted with 95 per cent ethanol. Two volumes of acetone were then added to the alcohol solution to precipitate the salts, which settle out in crystalline form. The salt crystals were also found to be slightly active. The observations indicate that the hormone adsorbs very readily. The hormone may be kept in a water solution in the refrigerator for some time without appreciable loss of activity. However, it is destroyed slowly when kept in a water solution at room temperature.

Physiology of the Eye-Stalk Glands

One of the basic problems in endocrine research is to determine the factors which affect the production of a hormone and the mechanism which regulates its release into the circulation. The standardization of the eye-stalk hormone has made it possible to investigate the physiology of the eye-stalk glands, for the amount of hormone in the stalks of animals maintained under special conditions can now be determined. The presence or absence of the hormone in the circulation can be determined quite easily by observing the states of the chief chromatophores with the aid of a microscope. This can be illustrated by the pigmentary reactions of *Uca* and *Palæmonetes,* the two animals chosen for this investigation. Perkins (1928) and Brown (1933) have shown that the contraction of the red and yellow chromatophores of *Palæmonetes* is due to the presence of the hormone in the blood stream, and that the expansion of these chromatophores results from the absence of the hormone. The situation is just reversed in *Uca* for in the brachyurans the eye-stalk hormone expands the melanophores and the erythrophores when it is circulating through the animal, while its absence results in the contraction of these chromatophores.

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[8] The alcohol-soluble material gave negative results when tested with Millon's reagent and with the Xanthaproteic test.

Palæmonetes

Forty specimens of *Palæmonetes vulgaris* of uniform size and weight were separated into four equal groups. One group was placed in a black vessel, another in a white, a third group in a yellow and a fourth in a blue. The vessels were then placed under the illumination of two 75-watt electric bulbs. The animals were supplied with a continuous current of fresh sea water, and left undisturbed for 8 hours. A fifth group of 8 animals were placed in total darkness for a day. At the end of 8 hours, the eye-stalks of each group under illumination were extracted with sea water and each of the 4 extracts assayed to determine the amount of hormone present. The eye-stalks of the animals placed in total darkness were extracted in darkness on the following day. The results of this investigation are listed in Table IV.

TABLE IV

Hormone content in the eye-stalks of Palæmonetes under various environmental conditions

Number of Eye-stalks Tested	Condition	Average Weight of Animals	*Uca* Units/E.S. of Palæmonetes
		grams	
20	Black-adapted	0.25	0.46
20	White-adapted	0.29	0.50
20	Yellow-adapted	0.30	0.49
20	Blue-adapted	0.25	0.47
16	Darkness (day)	0.28	0.25

This experiment was repeated three times and the same result was obtained in each case. The amount of hormone present in the eye-stalks of animals kept under illumination was twice that obtained from animals maintained in darkness. This is confirmatory of Kropp and Crozier's finding that stalk extracts of animals kept in darkness did not depress the growth rate of *Lupinus* as much as extracts made from animals exposed to light. It is also in agreement with the results of Kleinholz who found that extracts from animals kept in the darkroom produced weaker responses in the retinal pigments than extracts of animals under illumination. Equally significant is the finding that the amount of hormone in the eye-stalks of animals showing a continuous release of the hormone (white-adapted group) was the same as that of animals showing no, or a subminimal release of the hormone into the circulation (black-adapted group).

This situation becomes understandable when the functional cycle of an endocrine gland is considered. The normal physiology of an endocrine gland consists chiefly in synthesizing and storing a hormone, and finally releasing it into the circulation. These three processes may conceivably be controlled separately, or may be controlled uniformly or in part by the same mechanism. In *Palæmonetes*, as in some other crustaceans, the endocrine glands (Blutdrüse of Hanstrom, 1934) of the eye-stalk are innervated from the cerebral ganglia. The functional innervation of a gland would therefore afford a simple mechanism for the release of the hormone into the circulation.

The results listed in Table IV can be readily explained upon the basis of relative rates of hormone synthesis and hormone release. In darkness, there is no release of the hormone into the circulation as indicated by the inactive position of the distal pigment cells of the retina, and by the expansion of the red integumentary chromatophores (Brown, 1935*b*).[9] The rate of synthesis of the hormone must also be decidedly reduced as evidenced by the assays of the eye-stalks. In the presence of light, however, hormone synthesis is greatly increased, and this effect is produced regardless of the background over which the animals are kept. Hormone synthesis, therefore, is due to incident light and is not primarily dependent on reflected light. This accelerating effect of incident light may therefore be termed the primary effect.

The release of the hormone, however, is definitely brought about by light reflected from backgrounds such as yellow or white. This is shown by the contraction of the red and yellow chromatophores of the integument and by the inward migration of the distal cells of the retina under such conditions. The release of the hormone into the circulation is maximal. However, in the black-adapted animals there is no release or only a sub-minimal release of the hormone into the circulation as indicated by the expanded state of the red chromatophores.[10] Yet under these two conditions, the amount of hormone in the eye-stalks is the same.

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[9] The condition of the red pigment in *Palæmonetes* maintained in darkness does not seem to be definitely settled. Perkins (1928) stated that the red pigment was concentrated in complete darkness. Brown repeats this statement (1933) but later (1935*b*) is of the opinion that the red pigment is expanded in shrimps kept in darkness.

This situation is understandable if we consider that the white background reflex produces not only a maximal release of the hormone (and acceleration of synthesis due to the primary effect) but also a concomitant increase in the rate of synthesis so that there is a balance between a high rate of synthesis and a high rate of release. This balance must be attained since *Palæmonetes* retains a transparent hue for months if kept continually over an illuminated white background. If the white background reflex effected only a maximal release, one would reasonably expect that over a long period of white background-adaptation, the level

TABLE V

Hormone content during diurnal rhythm

Condition	*Uca pugilator*			*Uca pugnax*		
	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.	Phase	Number of Eye-stalks Extracted	*Uca* Units/E.S.
Retina extirpated 1 week previously						
Day, light..........	Black	10	0.98	Black	10	0.90
Day, dark..........	Black	8	0.94	Black	10	1.61
Night, light........	Pale	26	1.30	Inter.	18	1.00
Night, dark.........	Inter.*	10	0.96	Pale	8	0.91
Normal animals						
Day, light..........	Black	30	1.0	Black	10	0.95
Day, dark..........	Black	10	2.0	Black	10	0.97
Night, light........	Pale	10	1.0	Inter.	18	0.93
Night, dark........	Inter.	10	0.90	Pale	10	0.99
1 eye-stalk removed 3 days previously						
Day, light..........	Black	16	0.95			
Night, light........	Pale	16	0.95			

* The abbreviation "inter." is used to designate the intermediate condition in coloration between black and pale.

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[10] The hormone is probably released in amounts and at a rate which constitutes a sub-minimal stimulus for the integumentary chromatophores. Some release must occur because Kleinholz (1935) writes that the retinal pigments of *Palæmonetes* are under control of the eye-stalk hormone, and in animals kept on a black background the retinal pigments are in their active state. Hence, if the same hormone affects both the retinal cells and the integumentary chromatophores, it must follow that the amount of hormone released in illuminated black-adapted animals is minimal for the eye-pigments but sub-minimal for the body pigments.

of synthesis would be outstripped by the rate of release. The glands would therefore become exhausted and the animals would become dark due to chromatophore expansion. This apparently never occurs. In the black-adapted specimens, the release of the hormone is proceeding slowly and consequently, a balance is also obtained between a lower rate of synthesis and this lower rate of release. The fact that the amount of hormone in the eye-stalks is the same in both white-adapted and black-adapted specimens is due simply to the primary effect of incident light.

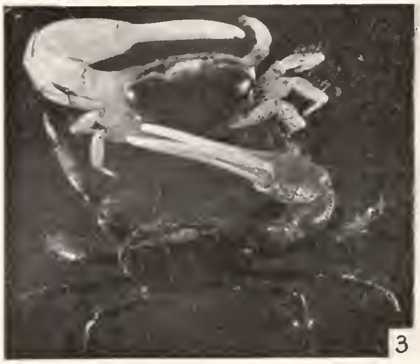
This interpretation of the physiology of the eye-stalk glands may be summarized as follows: Incident light (as opposed to light reflected from backgrounds) induces an acceleration of hormone synthesis but exerts only a sub-minimal release of the hormone into the circulation. This effect of light may be called the primary effect, and occurs in specimens placed on any background provided overhead illumination is present. The white background response, however, is due to the combination of the primary effect of incident light and of the effect of reflected light, which is to produce a maximal release of the hormone and a concomitant increase in the rate of synthesis.

Uca

Uca differs from *Palæmonetes* in that it undergoes a periodic change in color and that background adaptation is lacking. Both *Uca pugilator* and *pugnax* show a periodicity of color change. The diurnal rhythm of *Uca pugnax* was described by Megušar (1912). The animals are black by day, pale by night, and this daily cycle repeats itself regardless of background or of light intensity. I have confirmed these statements and have extended them to *Uca pugilator*. When the eye-stalks are removed the rhythm is permanently abolished (at least until regeneration of the stalks takes place) and the animals remain pale regardless of background or light intensity. Periodicity is therefore controlled by a rhythmical release of the hormone into the circulation, the release occurring every 12 hours.

Several animals (*Uca pugnax*) were maintained upon an illuminated white background from Oct. 20–Nov. 14, 1935. During the day they were jet black in color, but at 5:30 P.M. they began to pale so that at 7:00 P.M. all the animals were pale (lemon yellow in color). At about 8 A.M. on the following morning the animals turned black again. The same was true of specimens maintained from Oct. 20–Nov. 14, 1935, in total darkness. Background adaptation in *Uca* seems therefore to be lacking. Periodicity is the chief factor in its color change.

Complete extirpation of both stalks leads to permanent destruction of the rhythm. Extirpation of but one eye-stalk does not impair the periodicity. One eye-stalk is therefore sufficient for the continuance of normal chromatic activity. If the retinal portion of both eye-stalks is cut off cleanly by a sharp scalpel, the rhythm is likewise interrupted. Such animals when maintained in the light on a white background remain black during both day and night, for 3 or 4 days. Similarly prepared animals maintained in total darkness remained black during both day and night for 3 days. After this period of time the rhythm appeared again but was quite erratic for a day or two, after which it appeared to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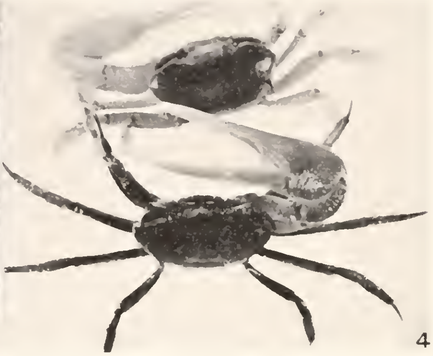
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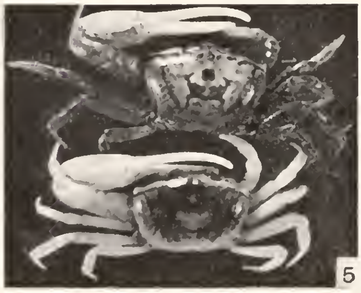
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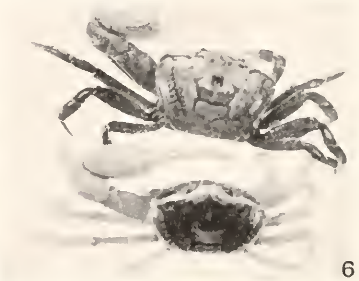
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Plate I

All photographs are from life and one-half life size.

Fig. 3. Two specimens of *Uca pugnax* photographed on a black background. Above, a specimen whose eye-stalks had been extirpated a day previously; and below, a normal animal in daylight.

Fig. 4. Same as Fig. 4, but photographed upon a white background.

Fig. 5. Two specimens of *Uca pugilator*. Above, a blinded animal 2 hours after injection of an extract of its extirpated eye-stalks. Below, an uninjected blinded specimen.

Fig. 6. Same as Fig. 6, but photographed upon a white background.

be normal again. During the past summer, I repeated some of these experiments with large numbers of animals (*Uca pugilator*). In constant light the periodic change in color occurred at 10–11 P.M. and at 9 A.M. Moreover, only about 70 per cent of the animals showed periodic changes.

The hormonal content of the stalks of selected animals under various phases of their diurnal rhythm was determined, and the results given in Table V. Animals of equal weights were used. The average weight of *Uca pugilator* was 2.5 grams, of *U. pugnax* 3.5 grams.

The results listed in Table V show that the amount of hormone present in the eye-stalks is the same whether the animals are in the pale phase of their rhythm or whether they are in the dark phase. There are some variations in the quantity of hormone extracted under different states, but these are statistically insignificant. As in *Palæmonetes,* the quantity of hormone extracted from the eye-stalks is the same in conditions during which the hormone is being continually secreted or is absent from the blood stream. This situation may be similarly interpreted. The diurnal rhythm of *Uca* is therefore an external expression of a diurnal release of the hormone into the circulation. During the 12 hours of release, the rate of production of the hormone may be increased to keep pace with its secretion into the blood.

The mechanism which controls this diurnal release differs from the release mechanism of *Palæmonetes.* The latter is definitely brought about by environmental factors (white background reflex), while in *Uca,* the diurnal release proceeds regardless of environmental factors. Furthermore, the theoretical increase in rate of synthesis is dependent on light in *Palæmonetes,* and is independent of light in *Uca,* since the diurnal rhythm proceeds in total darkness. The common feature shared by both these forms is that the release is probably controlled nervously. This is not as certain in *Uca* as it is in *Palæmonetes,* but the fact that the Blutdrüse in *Uca* are innervated and the observation that their normal physiology may be temporarily disturbed by destruction of the retinal portion of the eye-stalks seem to point towards the presence of a functional innervation. At any rate, it is difficult to imagine that the diurnal rhythm is an intrinsic property of the gland cells, for an inherent diurnal synchronism of all the cells in a gland would be somewhat surprising. Furthermore, if this conception is true, one would reasonably expect to find variations in the content of hormone during various phases of the rhythm since the glands would secrete during the day and synthesize during the night. The data in Table V show that this does not occur. It would be more satisfying to regard the blood glands as being regulated by a diurnal flow of nerve impulses. In either case, the origin of the diurnal rhythm is unknown, for one is now left without an explanation of the cause of a diurnal flow of nerve impulses. The intermediary step in the cycle is clear, however, for this is merely a diurnal release of the hormone into the circulation. The change in body coloration is but the external expression of this diurnal release.

A final experiment was performed to determine if a compensation of either hormone synthesis or release is made by the intact eye-stalk when its fellow is removed. One eye-stalk was removed from each of 32 specimens of *Uca pugilator* and 3 days later, the remaining eye-stalks were extracted and assayed. During this 3-day interval, the diurnal rhythm proceeded quite normally, the animals being maintained under illumination. The eye-stalks of 16 of these crabs were assayed during the day, the remaining 16 during the night. The results, tabulated in Table V, show that the amount of hormone in the remaining eye-stalk of such "one-eyed" crabs is the same during their dark phase as during their pale phase, and also the same, per eye-stalk, of normal crabs. Compensation, therefore, does not take place. One eye-stalk is quite sufficient to regulate chromatic activity when its fellow is removed.

Discussion

The interpretation just advanced should be considered only as a suggestion for it represents the first attempt to understand the normal physiology of the eye-stalk glands. It should also be stressed that the method of assay is sensitive enough to detect the difference between a concentration of X and one of 2X. Consequently, if slight variations do occur in the hormone content of the eye-stalks, these will not have been detected. Furthermore, inhibiting and activating systems may be present and play important parts in the biochemistry of the hormone.

It should also be emphasized that the theoretical considerations and the interpretations advanced are dependent upon the assumption that the glands in the eye-stalk are the chief if not the sole source of the manufacture of the hormone. This assumption is generally believed to be true, but certain recent observations are opposed to this notion. Hosoi (1934) found that extracts of the ventral nerve cord and male genitalia of *Penæus* produced chromatophore contraction when injected into blinded specimens of *Paratya,* showing that these extracts contained the chromatophorotropic hormone. Extracts of the stomach and muscles were also slightly active, but extracts of heart tissue were decidedly inactive. By a simple method Hosoi calculated that the eye-stalks were about one hundred times stronger in hormone content than the ventral nerve cord or genitalia. These results were criticized by

Kleinholz (1935), who attributed them to the presence of the hormone in the coagulated blood contained in these organs. Kleinholz's criticism seems to be well taken, but it is rather surprising that heart extracts were negative since these should also contain coagulated blood. At any rate, Hosoi does not describe the type of animal from which the extracts were made. If the extracts were prepared from shrimp showing the phase in which the eye-stalk hormone was being released into the circulation, Kleinholz's criticism would seem very justifiable and the importance of Hosoi's results would be uncertain. If the extracts were made from black-adapted specimens, or better, from animals whose eye-stalks were amputated some time previously, Hosoi's results could then be taken to indicate that organs other than the eye-stalks may produce the hormone. Brown (1933, 1935*a*) likewise states that extracts of the ventral nerve cord of *Palæmonetes* are active when tested on the chromatophores of blinded specimens. However, he gives no data concerning the phase of the shrimps from which his extracts were prepared nor of the potency of such extracts in relation to an extract of the eye-stalks. It would seem that Kleinholz's criticism of Hosoi's work would apply aptly to that of Brown. It has not been shown conclusively, therefore, by this type of experiment that other tissues may produce the hormone found so abundantly in the eye-stalks.

A second line of evidence bearing on this question has been advanced by Brown (1935*a*). Brown applied heat and electricity to the stubs of the eye-stalks in animals whose stalks were previously removed and observed that such stimuli induced rapid chromatophore contraction. This reaction was interpreted by him to be due to the excitation of endocrine glands of some region of the body outside of the eye-stalks by these heterologous stimuli (heat, electricity). However, this interpretation must be supported by the demonstration that the assumed endocrine glands in some region of the body are actually present, and that the contraction of the chromatophores following such stimuli is actually hormonal.

It can be concluded from this discussion that the eye-stalks are the chief source of the production of the chromatophore hormone. It can also be stated that none of the experiments already mentioned shows conclusively that tissues other than the eye-stalks may produce the hormone. The facts that under illumination blinded *Palæmonetes* remain steadily dark, and that blinded *Uca* remain continuously pale indicate that if other tissues, capable of forming the chromatophore hormone, are present they play an insignificant part in the ordinary chromatic physiology of these animals.

The interpretation of the mechanism of diurnal rhythm in *Uca* differs slightly from that advanced by Young (1935) for the cyclostome, *Lampetra*. *Uca* and *Lampetra* show many features in common. In each animal background adaptation is lacking. Each animal is pale during the night, and dark during day. Each responds to the loss of its chromatophore hormone by complete pallor, and each responds to the loss of its retinas by melanophore expansion, which is not as permanent in *Uca* as in adults of *Lampetra*. The diurnal rhythm of either *Uca* or *Lampetra* may proceed in total and enduring darkness. However, under constant illumination, the coloration of *Lampetra* remains steadily dark while the color rhythm of *Uca* may continue. To account for the periodic change in the coloration of the lamprey, Young suggested that the paired eyes in collaboration with the pineal glands affected the melanophores by a nervous inhibition of the secretion of the melanophore-expanding substance by the pituitary so that the animal becomes pale. This associates the active phase of the daily cycle with the appearance of the pale coloration of the lamprey, that is, with its nocturnal hue. The mechanism advanced for *Uca* places the active phase of the cycle with the appearance of its diurnal hue, but there appears to be no reason why Young's interpretation could not also apply to the situation in *Uca*. What actually happens in either *Uca* or *Lampetra* is the secretion of the melanophore hormone (assuming that we are dealing in terms of one melanophore substance in either case) into the circulation during the day, and its absence during the night. This daily cycle may be due to (1) a diurnal nervous stimulation of secretion, (2) a nocturnal nervous inhibition of the glands which, if not inhibited, would continue to secrete, or (3) to a combination of both. At present, it is difficult to prove which of the three possible mechanisms is correct. The interpretation that the active phase of the daily cycle in *Uca* was associated with the dark phase was made in consideration of the results obtained with *Palæmonetes*, in which the release of the hormone into the circulation is evidently due to a nervous stimulation of the glands.

In conclusion, it would be fair to outline briefly other possible explanations of the physiology of the eye-stalk glands in *Palæmonetes*. The explanation already advanced is based partly on the observations of Brown (1935*b*), which may not be entirely correct, and also takes into consideration the behavior of the retinal pigments on the basis that they are affected by the same hormone. Concerning the latter, there are no indications at present for the existence of two hormones, one for the body pigments, and one for the retinal cells. All that is known now is that the same extract which affects the body chromatophores also affects the retinal cells (Kleinholz, 1935). The key to the whole situation lies in the behavior of the red and yellow chromatophores in animals maintained in darkness. If Brown (1935*b*, p. 320, but see also p. 328) is correct in noting that the more usual condition of the red and yellow pigments of animals maintained in darkness was slight dispersion, and that a long sojourn (2–3 weeks) in darkness resulted in the same condition of these pigments as that occurring when animals were adapted to a red background (cf. p. 319, Brown, 1935*b*), there is little reason for postulating separate autocoids for the body and retinal pigments. Threshold differences would account for the various responses. If the earlier observation of Perkins (1928) that the red and yellow pigments are contracted in darkness is correct, the existence of separate hormones would be clearly indicated (because the chief body pigments would react completely independently of the retinal pigments in darkness and on an illuminated black background), and the entire problem would be greatly simplified. Secretion and synthesis of the retinal hormone would take place only under the action of light; in darkness, release would be abolished and synthesis reduced. For the chief body pigments, it would have to be assumed that the gland produces and releases the hormone continuously, that synthesis is accelerated by incident light regardless of background, and that release is inhibited by a black background. The latter process seems theoretically difficult, for a white background reflects while a black background absorbs light. Finally, the stimulus for release or for inhibition of release may depend on the excitation of certain portions of the retina by a particular ratio of incident to reflected light. All these possibilities are well worth investigation.

Summary

A method for the standardization of the crustacean eye-stalk hormone on the blinded fiddler crab, *Uca*, has been described. The *Uca* unit has been defined as the amount of hormone contained in 1 cc. of solution, 0.05 cc. of which when injected into each of 15 specimens of *Uca pugilator* blinded 2 days previously produces a response whose average duration is about 5.0 hours. The response is measured as the amount of time intervening between the injection of the hormone and the time at which the animals again become pale, an interval during which the melanophores expand, remain expanded for some time, and finally contract. The hormone content in the eye-stalks of various crustaceans was determined. A method for the extraction and purification of the hormone has been described, and some chemical and physical properties of the hormone have been listed.

The amount of hormone extracted from the eye-stalks of *Palæmonetes* is the same regardless of whether the hormone is secreted continuously into the circulation, or whether it is continuously absent, conditions which are brought about by illuminated white and black surroundings respectively. In darkness, there is no release of the hormone into the blood, and a very low content of hormone in the eye-stalks, approximately half that obtained from the stalks of illuminated animals. It is postulated that light, regardless of background, causes an acceleration in hormone synthesis, and that light depending on certain backgrounds such as white, causes a maximal release of the hormone into the circulation with a concomitant increase in rate of production of the hormone. The diurnal color rhythm of *Uca* is an external expression of a diurnal release of the hormone into the circulation. Both release and synthesis are independent of environmental conditions, and it is suggested that they are controlled by a diurnal discharge of nerve impulses from the C.N.S. This discharge, during the day, would exert a 12-hour release of the hormone with a concomitant increased rate in production, the absence of the discharge during night would cut off release and slow down rate of synthesis.

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CALCIUM REDUCTION AND THE PROLONGATION OF LIFE IN THE EGG CELLS OF ARBACIA PUNCTULATA

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INTRODUCTION

Most investigators of the unfertilized egg cell have, naturally, been concerned with the problems of fertilization and localization. I do not believe that the egg has often been thought of as a living organism with a definite life span, quite independent of its fertilizable life. And yet, freshly shed eggs which cannot be fertilized are undoubtedly alive. Occasionally their fertilizability may be restored (Lillie, 1912). Also, aged eggs which do not respond to sperm are not necessarily dead (Woodward, 1918). In a study of longevity it is important that there be no confusion between the living condition and the ability to receive sperm and to cleave. The two are not synonymous and the test of fertilizability is not the test for life.

It seems of interest to point out that with regard to longevity the unactivated egg cell is quite different from other unicellular organisms, like the protozoans, in that its life span is very definitely limited; and, as Jacques Loeb (1913) has suggested, death of the unfertilized egg is perhaps the only case where we can be sure that it is due to " natural causes " and not to avoidable injuries. But the concept developed by Loeb and Northrop (1917) for the Metazoa, that natural death is connected with compound character, obviously falls down here where there is no organ differentiation.

This paper is concerned with the prolongation of life in the unfertilized egg cells of *Arbacia punctulata.* It seems reasonable to assume that factors which prolong life are involved also in natural death.

METHODS

Shed eggs, 98–100 per cent of which were able to cleave, were used. The eggs were washed in one or two changes of 250 cc. of filtered sea water and then concentrated into 50–60 cc. by settling. Ten-cc. samples were added to 200 cc. of the experimental solutions in finger-bowls. These dishes, covered, were stored for the duration of an experiment on a running sea-water table in subdued daylight. The temperature for the whole series of experiments varied between 20 and 21.5° C. Old solutions were siphoned off and the concentrated eggs transferred into fresh solutions in clean dishes at least once and sometimes twice in 24 hours. As dead eggs often adhere to the bottom of the dish, an unavoidable error was introduced in calculating percentage dead at any time after the first change. However, this error does not affect the end-point when no living cells remained and it was minimized by taking readings before changing solutions.

Merck C.P. or " Reagent Grade " chemicals were used and, where obtainable, " For Biologic Work." NaCl and KCl were made up in 0.53 M concentration; $MgCl_2$ and $CaCl_2$ in 0.29 concentration.[1] NaCl, KCl and $MgCl_2$ were mixed in the proportions of 100/2½/10 by volume, which is approximately the normal concentration of the cations in sea water. In the initial experiments (represented in Fig. 1), sea water was diluted with this Ca-free medium. Thus, to obtain ⅙ calcium concentration, 500 cc. of the Ca-free medium was added to 100 cc. of sea water. The concentrations used were ⅔, ⅓, ⅙ and 1/12 calcium besides normal calcium in sea water as a control. The hydrogen ion concentrations of these solutions, measured colorimetrically, were respectively pH 8.3, 8.2–3, 7.6, 7.4 and 8.3. It is apparent that the buffering action of sea water prevented any significant change in pH down to a dilution of one part of sea water to 5 parts of Ca-free solution.

In later experiments sea water was diluted with five-sixths of artificial medium made up to contain NaCl, KCl and $MgCl_2$ in proportions as above, plus varying amounts of $CaCl_2$. Except for the controls in sea water the pH of all of these was 7.6. Solutions made up in this way were used in the experiments represented in Fig. 2.

In the experiments of Fig. 3 isosmotic dextrose (0.95 molal) was substituted for NaCl, KCl, and $MgCl_2$, and the solutions were made up with 1 part sea water, 5 parts isosmotic dextrose and sufficient isosmotic $CaCl_2$ to give the concentrations of calcium indicated. For example, to obtain 2.6 × Ca the following: 150 cc. sea water, 750 cc. 0.95 molal dextrose, 91 cc. 0.29 molar $CaCl_2$.

Concentrations of calcium are calculated on the basis of 0.428 gram/liter, which is normal for sea water (Page, 1927). This point is unity on the graphs.

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[1] Theoretically 0.34 M $CaCl_2$ is isotonic to sea water, but by empirical test Mr. Leon Churney of the University of Pennsylvania has found 0.29 M to be more nearly isosmotic with the *Arbacia* egg.

For observation, at least 100 eggs, usually more, were counted to obtain the percentage disintegrating. These values were plotted against time in hours and time for 50 per cent to disintegrate obtained from the curves. In some experiments samples were fertilized at each observation.

The Prolongation of Life

In a study of the effects of calcium on subsequent cytolysis in distilled water, the results of which have already been briefly reported (Schechter, 1936), it was found that exposure for several hours to Ca-free media produced no noticeable deterioration of *Arbacia* eggs and they were able to cleave when fertilized in sea water. On the other hand, 25 minutes in isosmotic $CaCl_2$ resulted in the disintegration of 40 per cent (23 out of 58 counted). After one hour and nine minutes none survived. Cleavage was reduced to zero after 15 minutes and this effect could not be reversed by an equal period of washing.

Table I

Effect of dilution of sea water with Ca-free medium on prolongation of life. Upper figures in horizontal rows percentage disintegrated, lower figures number of eggs counted.

Age in Hours	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
16½	3.7 108	2.4 125	0 105	1.4 146	0 132
22	14.3 112	9.4 139	2.5 121	0.7 139	0.4 241
39½	48 115	48 159	4.4 113	4.6 152	0
48	84 134	85 112	4.9 143	0.6 177	0
66¼	100	100	68 114	10 121	0.95 210
90½			100	74 133	17 147
115				100	82–100 218

The lethal influence of pure $CaCl_2$, not shown with sodium and potassium chlorides within the time indicated above, led to the work in which calcium concentration was varied as described under methods. To illustrate the nature of the observations the data of one experiment are presented in Tables I and II. The eggs were obtained at 2:13 P.M. August 4 from an urchin collected and brought to the laboratory at 11:10 A.M. Ninety-eight per cent cleaved on test. After washing in two changes of 250 cc. of sea water, and concentrating into 50 to 60 cc., a 10-cc. sample was placed in each of the experimental solutions at 4:30 P.M. An equal sample in sea water served as a control.

In Table I the top figure at each age is the percentage disintegrated, i.e., visibly in bad condition. The lower figure is the number of eggs upon which the percentage is based. Table II represents the percentage which cleaved as a fraction of those which appeared to be in good condition. The lower figures are numbers of eggs upon which the percentages are based. These data exclude disintegrated eggs and show physiological condition in the survivors, insofar as fertilizability and cleavage measure such condition. Table III is derived from Tables I and II, by the product of percentage of cleavage and percentage of live eggs, and is appended to give an indication of the extension of fertilizable life in terms of the total population at the beginning.

TABLE II

Effect of dilution with Ca-free medium on prolongation of fertilizability in surviving eggs. Upper figures percentage cleaved, lower figures number of eggs counted.

Age in Hours at Fertilization	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
17	93	94	81	89	87
	86	96	102	105	99
22½	71	50	69	61	60
	61	102	96	105	114
40¾	65	50	67	59	80
	62	58	116	78	84
48½			78	67	58
			101	104	127
67			22	62	84
			41	109	102
91				2	45
				51	89

The photographs in Plate I are taken from the above-described experiment. Photographs 1 to 4 are of eggs aged $39\frac{1}{2}$ hours in sea water, $\frac{2}{3}$ calcium, $\frac{1}{3}$, and $\frac{1}{12}$ calcium. They show progressively fewer disintegrated with greater lowering of the normal calcium concentration. Photographs 5 to 8 are, respectively, samples of the same, fertilized at $40\frac{3}{4}$ hours of age. Photographs 9 and 10 contrast the condition at $66\frac{1}{4}$ hours of age of unfertilized eggs in sea water (all of these having disintegrated long before) and in $\frac{1}{12}$ calcium. The latter, fertilized when 67 hours old, show a very high percentage of cleavage (Photograph 11) and even when 91 hours old (Photograph 12) the cleavage percentage is high and early cleavage, at least, is normal.

TABLE III

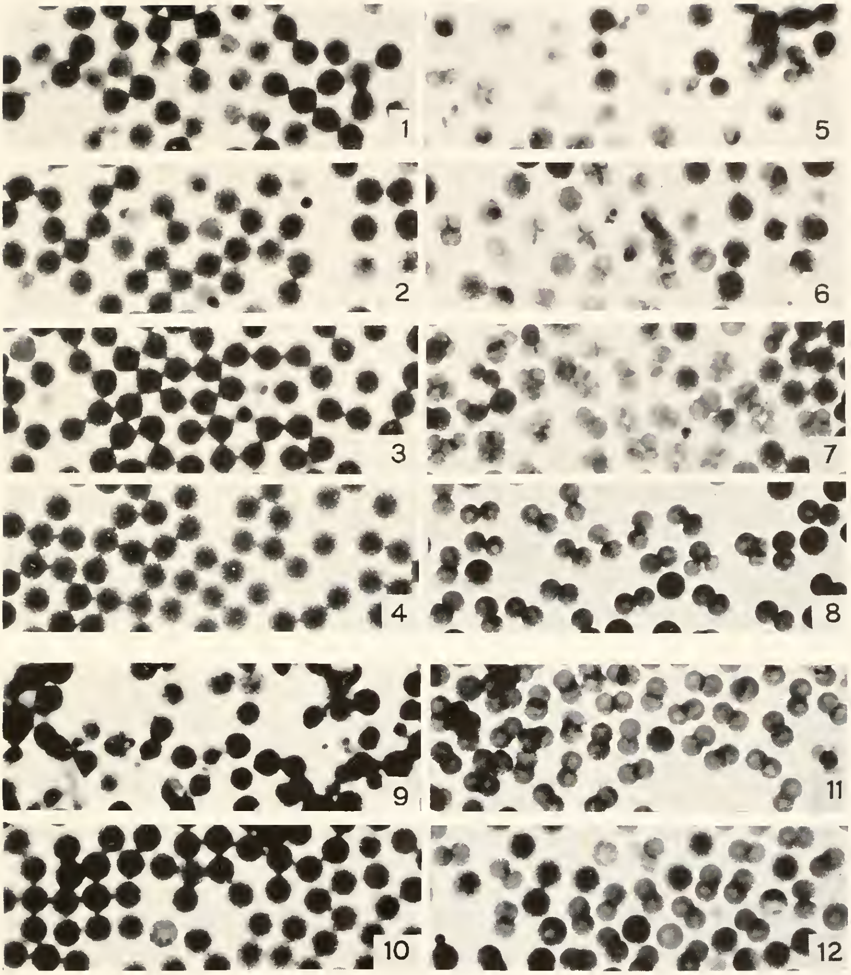
Effect of dilution with Ca-free medium. Surviving percentage of total population able to cleave.

Age in Hours at Fertilization	In Sea Water	In 2/3 Ca	In 1/3 Ca	In 1/6 Ca	In 1/12 Ca
17	89	91.8	81	87.7	87
$22\frac{1}{2}$	61	45.3	67.3	60.5	60
$40\frac{3}{4}$	33.8	27	64	56.3	80
$48\frac{1}{2}$			74	66.6	58
67			7.05	56	83
91				0.52	37.4

Seven experiments of the kind just described were performed. In three the eggs of two or three females were combined, giving a total of eight and in four others single lots of eggs were used. The results of these twelve are presented in Fig. 1. Calcium concentration, in terms of that normal to sea water as unity, is plotted on the ordinate axis and time for survival of 50 per cent of the population at various concentrations on the abscissa. Full data support all points except at $\frac{1}{12}$ calcium where measurements are available only from 5 lots of eggs.

It is apparent from the graph that the span of life increased steadily upon dilution with Ca-free medium. From 30 hours in sea water, longevity rose 300 per cent to 90 hours at $\frac{1}{12}$ the normal calcium (and other changes in this solution to be brought out later).

Four experiments were carried out with a constant fraction of sea water, calcium variation being obtained by adding $CaCl_2$ to the artificial medium as described under methods. In this way the pH was constant and differential dilution of any possibly toxic constituents of sea water was also avoided. The results are expressed in Fig. 2, and for comparison the range from 0.88 to 0.167 calcium is shown on Fig. 1 by a dotted



EXPLANATION OF PLATE I

PHOTOGRAPHS 1–4. Eggs aged for 39½ hours in normal calcium (sea water), ⅔, ⅓, and 1/12 calcium respectively.

PHOTOGRAPHS 5–8. Cleavage in eggs fertilized after 40¾ hours in above solutions, respectively.

PHOTOGRAPH 9. Eggs aged 66¼ hours in sea water.

PHOTOGRAPH 10. Eggs aged 66¼ hours in 1/12 calcium.

PHOTOGRAPH 11. Eggs aged in 1/12 calcium, fertilized when 67 hours old.

PHOTOGRAPH 12. Eggs aged in 1/12 calcium, fertilized when 91 hours old.

line. In Fig. 2 the larger dots represent the average of observations on four lots of eggs and the smaller dots those of two. The pH for all points was 7.6 except that of sea water which was 8.3.

In Fig. 3 are given the averaged results of two experiments with $^{1}/_{6}$ sea water plus $^{5}/_{6}$ dextrose and different amounts of isosmotic $CaCl_2$ added. The measured pH varied at random from 6.7 to 7.0.

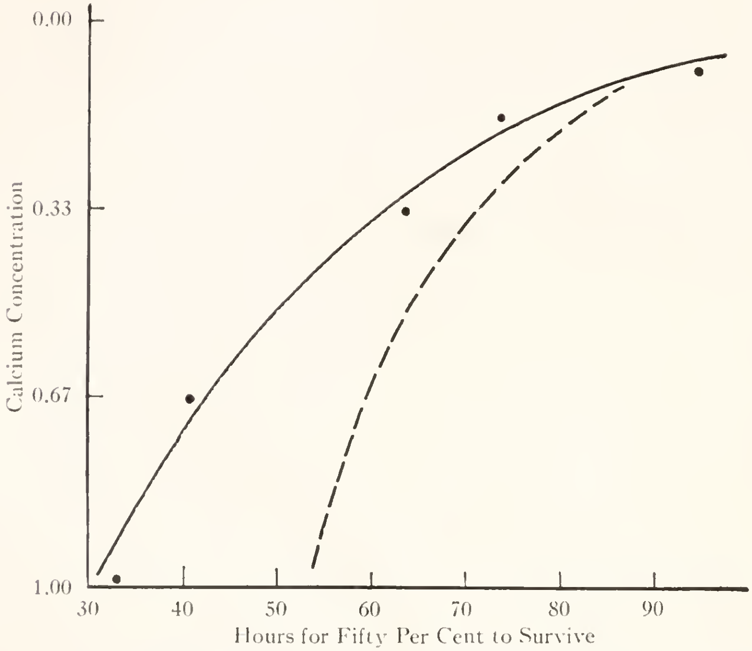


FIG. 1. Effect of dilution of sea water with isosmotic Ca-free salt solution on the longevity of unfertilized *Arbacia* egg cells. Unity on the ordinate represents undiluted sea water. pH is 8.3 except 7.6 in $^{1}/_{6}$ sea water and 7.4 in $^{1}/_{12}$.

DISCUSSION AND CONCLUSION

The results leave no doubt, I believe, that decrease in calcium prolongs the life of the *Arbacia* egg. With a step-by-step elimination of other variables this phenomenon becomes increasingly clear.

Divergence between Figs. 1 and 2 in the range of calcium concentration close to that of sea water and the fact that the control group falls off the curve on Fig. 2 may be accounted for on the basis of pH. Smith and Clowes (1924) have already shown that the optimal point for longevity is below the pH of sea water. But even with constant pH, as in Fig. 2, the effect of decreased calcium is evident. The steepness of the curve between calcium concentrations 1 and 2.6 may mean that in this range pH becomes the limiting factor for longevity, until the lethal action of calcium is again sufficient to bend it toward the left. It may be that these two factors are not mutually exclusive but that acidification acts through the mechanism of calcium by a reduction in

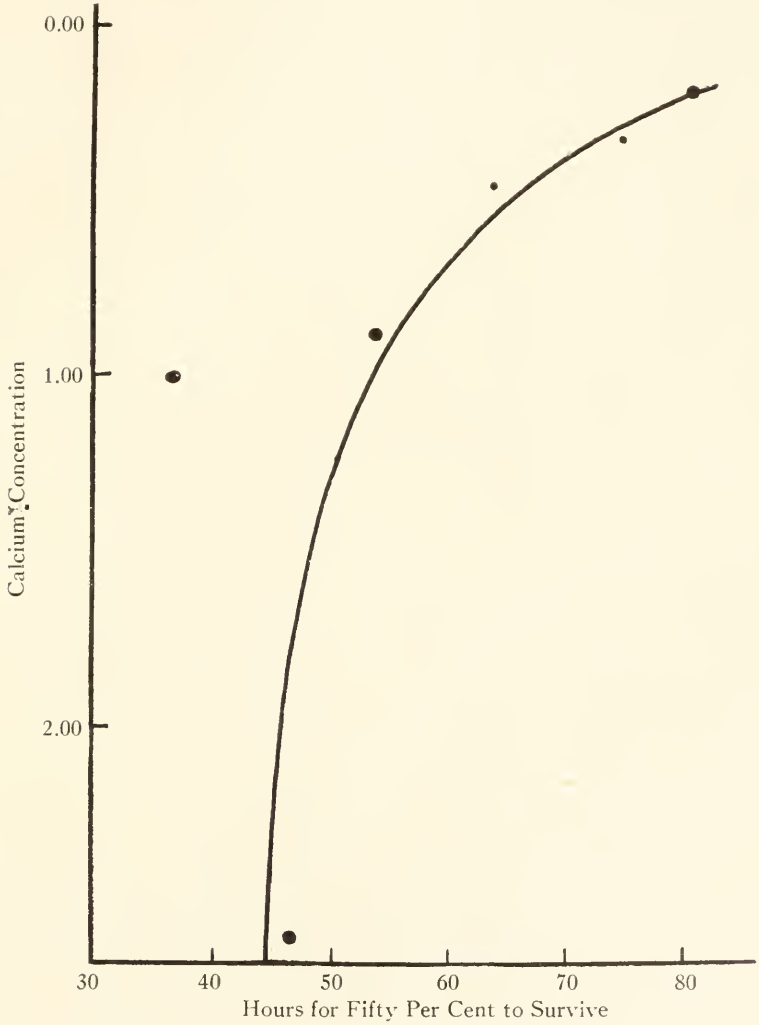


Fig. 2. Effect of calcium reduction on longevity of *Arbacia* eggs in $\frac{1}{6}$ sea water + $\frac{5}{6}$ Ca-free solution. pH constant at 7.6. Calcium varied by addition of 0.29 molar $CaCl_2$. Point at unity on the ordinate represents sea water control, pH 8.3.

the base-binding power of cellular materials as they approach their isoelectric points.

The use of a non-electrolyte in the experiments of Fig. 3 excludes the possibility that increase in sodium, potassium and magnesium, which was colligative with decrease in calcium in the other experiments, is responsible for the results. Here there was no electrolyte variable but calcium; and it may perhaps be significant that there appears to be a most nearly straight-line relationship. Actually, however, the curve may be S-shaped, but unlike that of Fig. 2, swinging quickly toward the ordinate axis in the absence of ions antagonistic to calcium.

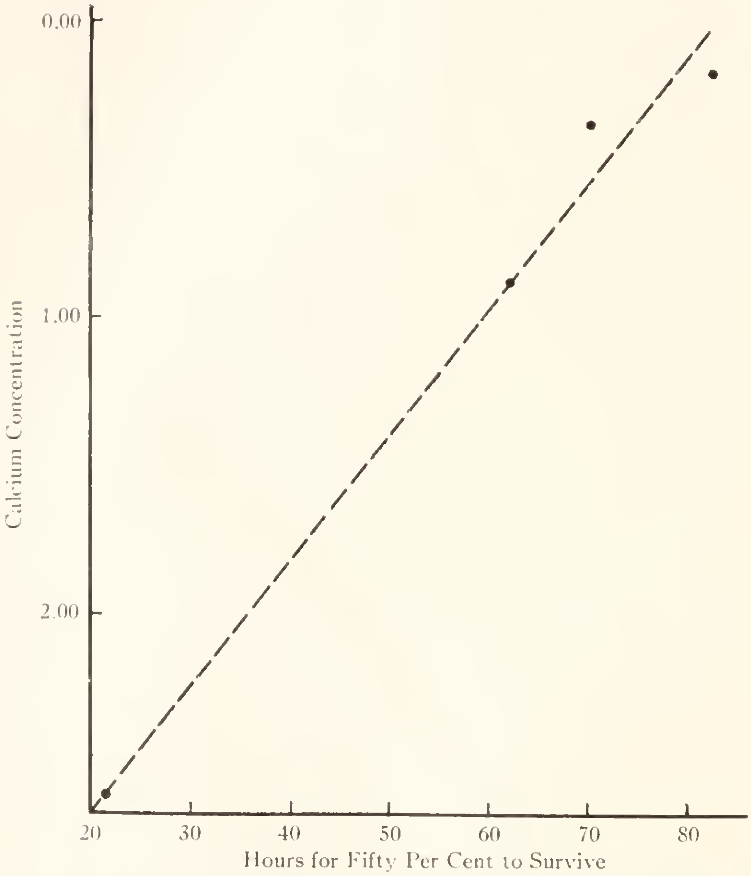


Fig. 3. Effect of calcium concentration on longevity of *Arbacia* eggs in $^1/_6$ sea water + $^5/_6$ 0.95 molal dextrose. pH ± 6.9. Calcium varied by addition of isosmotic $CaCl_2$.

The influence of dextrose is, of course, a disturbing factor. In pure dextrose the permeability of the egg rises, but 0.00005 M $CaCl_2$ is sufficient to keep it at sea water level (McCutcheon and Lucké, 1928). The lowest concentration of $CaCl_2$ in my experiments with dextrose is fifty times this amount. Here, however, calcium is antagonized by sodium and potassium (in $\frac{1}{6}$ calcium solution). At 0.34 concentration there is an excess of 0.002 M free calcium; and at this point, with permeability change presumably ruled out, life duration is 350 per cent greater than at 2.6x calcium, as seen from the graph. It must be kept in mind, however, that the permeability studies were made on fresh material exposed to the experimental solutions for short periods of time. The usefulness of the results in this discussion assumes transferability to my experimental conditions involving long exposure.

As this writing is in progress an advance abstract has appeared in which Whitaker (1936) reports that 1 per cent dextrose and 1 per cent alcohol in sea water, each prolongs the fertilizable life of *Urechis* eggs. Although it is premature to comment in the absence of a complete account, may I point out (Heilbrunn, 1934) that with low free calcium (which is probably the condition in the interior of an egg) 1 per cent alcohol (and 1 per cent ether) prevents the surface precipitation reaction, in which calcium is significantly involved. Also, Gray (1926) found that sugar and alcohols act like calcium in their stabilizing effect on the matrix of *Mytilus* gill tissue. It may, therefore, be that the action of alcohol and of sugar on the *Urechis* egg is effected through a change in the calcium equilibrium.

If calcium is a factor in determining longevity in *Arbacia* egg cells, we might expect a similar mechanism within the body of the sea urchin, where the cells may live for a prolonged period. Analysis of the cœlomic fluid[2] of female sea urchins showed a calcium content of 0.395 mg./cc. against 0.41 mg./cc. in sea water. On the basis of the data of this paper the difference, of about 3½ per cent, is insufficient to account for longevity of eggs within the animal. However, calcium content in the gonad may be lower than that of the cœlomic fluid. No direct evidence is available but Heilbrunn (1928, p. 148) has reported that eggs taken from the ovary are much more resistant to cytolysis in isosmotic $CaCl_2$ than if they are first equilibrated with the salts of sea water. Therefore, regardless of the negative results from cœlomic fluid analysis, a calcium mechanism for longevity of ovarian eggs is a possibility.

Finally, because of my interest in bioelectric phenomena (Schechter, 1934), may I point out that electrical potentials are correlated with calcium changes within the range of these experiments (Dan, 1936). Electrophoretic potential rises from — 30 to — 20 millivolts as calcium concentration is reduced.

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[2] The analysis was made by Daniel Mazia of the University of Pennsylvania to whom I wish to extend my thanks.

SUMMARY

1. At ± 21° C. in sea water 50 per cent of the eggs of *Arbacia punctulata* remained undisintegrated for about thirty hours.

2. With decrease in calcium to $^1/_{12}$ that found in sea water and a drop in pH from 8.3 to 7.4, 50 per cent of the eggs survived for ninety hours.

3. With pH constant at 7.6, 50 per cent of the eggs survived for 53 hours in an artificial solution with normal calcium concentration and for 90 hours in $^1/_{12}$ calcium.

4. With other electrolytes kept constant by the use of dextrose solution 50 per cent of the eggs survived for 20 hours in 2.6x the normal calcium and for more than 80 hours in $^1/_{12}$ concentration.

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THE EFFECT OF ULTRA-VIOLET LIGHT UPON EARLY DEVELOPMENT IN EGGS OF URECHIS CAUPO[1]

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Introduction

During the summer of 1936 while at the Hopkins Marine Station, Pacific Grove, California, the writer observed the effect of ultra-violet radiation upon the early development in eggs of *Urechis caupo,* Fisher and MacGinitie (1928*a,* 1928*b*). The eggs of this marine worm are immature when shed, hence maturation ensues after fertilization. The present paper gives results which show the effect of ultra-violet radiation upon maturation and first cleavage, together with the effect upon the rate of these early stages. The study does not aim at a physical analysis of radiation effects, but rather, at the qualitative effects of radiation upon maturation and first cleavage.

Material and Methods

The worms were collected at Elkhorn Slough, Monterey Bay Region, California. To insure the use of gametes in best condition the usual practice was to collect animals as often as tide conditions were favorable. Experimental animals were kept no longer than three weeks. They gave a yield of eggs and sperm of high fertilization capacity throughout the entire period of the work. Quantities of gametes were removed from the worms (males and females were kept in separate aquaria) when needed. Eggs were placed immediately into dishes containing 250 cc. sea water and dry sperm were kept in covered Syracuse watch glasses.

Irradiation was by means of an Analytic Model Quartz Lamp, Hanovia, which operated on a 110-volt circuit, alternating current, 60 cycles, 5 amperes.

All handling of eggs and sperm was done at a controlled temperature of 20° C. (plus or minus 2.5° C.). For regulation of the temperature of the sea water into which the eggs were placed several water-baths were used. Within each bath a thin-walled, flat-bottomed, glass dish was supported. This dish held the eggs and adjacent to them was placed the bulb of a calibrated thermometer.

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[1] This work has been supported in part by a grant from the Committee on Radiation, National Research Council.

Conditions were standardized as much as possible. The ultra-violet lamp was allowed to burn for 10 minutes before exposures were made. During this time traces of ozone were removed and the light reached its maximum intensity. Radiations were made at a distance of 30 cm. from the source of the light and the unfertilized eggs were given non-lethal exposures to the full spectrum of the mercury arc, for periods of 30, 35, 40, 45, 50, 55, and 60 seconds.

An experiment consisted of placing eggs whose controls gave 99 per cent to 100 per cent polar body extrusion and 97 per cent to 100 per cent first cleavage, in a thin layer on the bottom of a dish filled with sea water to a depth of 2.5 mm. This dish was placed in the constant temperature bath under the ultra-violet light and the eggs were irradiated. The dish was then removed and placed in a constant temperature bath fitted to the microscope, where the eggs were observed for five minutes for activation (development of fertilization membranes) by the radiation. When no activation was found the eggs were inseminated with a standard sperm suspension (one drop of dry sperm from a capillary pipette to five cc. sea water) and allowed to develop. The eggs were examined later for the extrusion of polar bodies and for first cleavage. Counts were made of the number of eggs in 100 which extruded first and second polar bodies, and of the number of eggs in 100 which showed first cleavage. These counts were repeated 19 times for each group of irradiated eggs. Twelve sets of experiments were made so that the data given below are for 24,000 eggs at each exposure.

The time-lapse was measured also, from the time of the insemination of the eggs to the time of the beginning of the particular stage considered. The time-lapse measurements for polar body extrusion were made on the reacting half of 10 eggs in the field of the microscope at the time of observation and the criterion was that moment when the polar body was sufficiently extruded to be identified unmistakably at the periphery of the egg. In cases where eggs were irradiated for 50, 55, or 60 seconds it was not possible to measure the time-lapse for the reacting half of 10 eggs because of the small number of polar bodies which could be found at the periphery of the eggs. I therefore measured the time-lapse for individual eggs and considered the average of the measurements made in the 20 counts as the time-lapse measurement. The criterion for first cleavage was that moment when the egg was elongated unmistakably at or in one or more axes (according to the regularity of cleavage) and the cleavage furrow could be seen as a thin, shining line which was especially clearly defined with the type of illumination used. The time-lapse was measured for the reacting half of 10 eggs in the field of the microscope, but at exposures of 50, 55, or 60 seconds when there were very few cleaving eggs it was necessary to measure the time-lapse for individual eggs and to take the average of the measurements made in the 20 counts as the time-lapse from insemination to first cleavage.

THE EXPERIMENTS

The extrusion of polar bodies and the first cleavage of the egg, together with the time after insemination at which these phenomena occur, are affected when the egg is irradiated before fertilization. The action of ultra-violet light upon these early stages in development is reported as the percentage of extrusion of polar bodies and cleavage, and the effect upon the rate of reaction is reported as the percentage increase in the time-lapse for each stage. The results are presented.

TABLE I

Percentage of polar body extrusion and first cleavage in eggs of *Urechis caupo* exposed to ultra-violet light for various lengths of time at a distance of 30 centimeters.

Length of Exposure (seconds)	First Polar Bodies	Second Polar Bodies	First Cleavage
30	91.3	88.9	73.1
35	78.4	73.9	57.7
40	63.8	55.1	40.7
45	31.4	18.5	14.0
50	9.5	2.8	5.2
55	4.7	1.4	4.2
60	2.3	0.68	2.5

All controls showed 99 per cent to 100 per cent polar body extrusion and 97 per cent to 100 per cent first cleavage.

The percentage is based upon the average of 12 experiments in each of which 2,000 eggs were counted.

Percentage Extrusion of Polar Bodies and Percentage Cleavage in Irradiated Eggs

From the results shown in Table I it is apparent that extrusion of polar bodies and the first cleavage of the egg are suppressed when eggs are irradiated with ultra-violet light. Different exposures varying from 30 to 60 seconds, with an increase of five seconds for successive durations of exposure, produce increasing suppression of the developmental stages until the longer periods of exposure are reached when there is a practically total suppression. Average percentages for all irradiations are presented for each duration of exposure which show unmistakably that the suppression of polar body extrusion and of first cleavage varies directly with the length of exposure. Since the data are based upon observations and counts of large numbers of eggs (the average percentages are for 24,000 eggs) the evidence is fairly conclusive. These results on the suppression of polar body extrusion confirm observations of Just (1933) for eggs of *Nereis limbata*. From the protocols of this observer the evidence shows that radiation effects were so pronounced that essentially a total suppression of polar body extrusion was caused when eggs were irradiated for 60 seconds at a distance of 25.5 cm. from the lamp. Just reported similar results when eggs were given longer exposures at different distances from the lamp.

TABLE II

Effect of ultra-violet light upon the time-lapse from insemination to polar body extrusion and to first cleavage in eggs of *Urechis caupo* exposed for various lengths of time at a distance of 30 cm. Percentage increase in time-lapse equals

$$\frac{\text{time-lapse in radiated eggs} - \text{time-lapse in control}}{\text{time-lapse in control}} \times 100.$$

Stage	Length of Exposure (seconds)	Time-lapse in Radiated Eggs (minutes)	Time-lapse in Control (minutes)	Per Cent Increase in Time-lapse
First polar bodies	30	30.0	29.8	0.67
	35	31.2	28.5	9.47
	40	32.1	28.3	13.43
	45	34.8	28.6	21.68
	50	38.3	27.5	39.27
	55	41.3	28.3	45.94
	60	42.3	28.6	47.90
Second polar bodies	30	44.3	42.3	4.73
	35	46.4	42.6	8.92
	40	47.3	42.3	11.82
	45	52.9	43.8	20.78
	50	55.6	42.1	32.07
	55	57.7	42.7	35.10
	60	59.7	42.2	40.76
First cleavage	30	69.2	67.4	2.67
	35	70.1	67.2	4.31
	40	70.3	68.0	3.38
	45	75.2	68.9	9.14
	50	82.5	69.2	19.22
	55	84.0	68.7	22.27
	60	89.2	68.5	30.22

Each time-lapse measurement represents the average time from insemination to the particular stage and is based upon data for the reacting half of 24,000 eggs observed at each exposure save 50, 55, and 60 seconds. At these exposures the average time-lapse was taken for the number of eggs in 24,000 which showed the particular stage.

Percentage of Increase in Time-lapse from Insemination to Polar Body Extrusion and First Cleavage

The data in Table II show the effect of ultra-violet radiation upon the rate of the early developmental stages in eggs of *Urechis caupo*. Time-lapse measurements for eggs irradiated at different exposures were compared with similar measurements in controls and the relation between the time-lapse from insemination to a particular stage in both the experimental and the control eggs was given as the percentage increase in time-lapse. The data show that wherever a developmental stage was affected by radiation the reaction time of the egg (time-lapse from insemination to the stage) was retarded. At the comparatively short lengths of exposure the percentage increase in the time-lapse was small. Each increase in length of exposure caused an increase in the percentage increase in time-lapse. The steady increase in the time-lapse from insemination of the egg to a particular stage as indicated in Table II may be regarded as evidence that irradiation of eggs not only affects certain stages in development but also affects the reaction rate of the eggs.

DISCUSSION

Suppression of polar bodies and of cleavage by such agencies as exposure to extremes of the viable range of temperature for eggs, treatment with hypotonic sea water, or subjection to the action of narcotizing substances, before or after activation, has been reported by many investigators. The data reported here suggest ultra-violet light as a most effective agent for suppression of early developmental stages.

The exact nature of the action of ultra-violet radiation is not definitely known. An important factor appears to be the extent of the absorption of radiant energy by the inner protoplasm of the egg and by the superficial layer, each of which is a site of complex reactions which underlie morphological changes and developmental processes. Evidence presented by Redfield and Bright (1921) and Just (1933) shows an alteration of the initial changes involved in the cortical reaction in the egg of *Nereis* fertilized after exposure to ultra-violet light. Another type of evidence is given by Tchahotine (1921*a*), who correlated local centers of injury produced in the peripheral layer of sea urchin eggs with permeability changes. Tchahotine (1921*b*) further pointed out the probability of the coagulation of the colloids of the superficial layer of the irradiated sea-urchin egg. While eggs of *Urechis caupo,* unlike eggs of *Nereis limbata,* extrude no jelly following fertilization and show no visible alteration of the superficial layer other than the separation of a tough, pellicle-like membrane (see Chase, 1935) from the vitellus of the egg which develops into the fertilization membrane, it is possible that certain changes take place in the egg cortex which are affected by radiation as in the case of other species of eggs. A serious alteration of the physical and chemical properties of the peripheral layer of the eggs conceivably may be a factor in the suppression of polar body extrusion in eggs which are radiated before they are fertilized.

On the other hand, the developmental stages which have been studied are closely related to various phenomena which occur deep within the egg. Of these the viscosity changes are the most widely studied. While no attempt was made to observe the effect of radiation on such changes in eggs of *Urechis caupo*, evidences of the effect of ultra-violet radiation upon viscosity of protoplasm of other eggs is reported. Of these investigations the most significant for this particular problem are the observations on the egg of *Ascaris*, which falls in the same category with the egg of *Urechis* with respect to the time at which the egg may be fertilized. Schleip (1923), in the course of observations on the effect of ultra-violet radiation on morphological components of *Ascaris* eggs, reported increased viscosity. Similarly, Ruppert (1924) centrifuged radiated eggs in his studies on the effect of ultra-violet light upon different stages in the development of eggs of *Ascaris* and his results indicate increased viscosity. Such changes alone may be associated with marked inhibition of phenomena which underlie maturation and cleavage processes and the resulting suppression of these stages in development.

A cytological study of the irradiated eggs is being made and it may give significant evidence on the effect of ultra-violet light upon morphological changes in the eggs.

SUMMARY

1. When unfertilized eggs of *Urechis caupo* are exposed to ultra-violet light for different lengths of time, then fertilized, polar body extrusion and first cleavage are suppressed. In addition to the suppression of these stages radiation causes an increase in the reaction rate of the egg which bears a direct relation to the length of exposure.

2. Possible factors in the suppression of these stages in maturation and first cleavage are the alteration or probable injury of the egg cortex and accompanying changes in its chemical and physical properties, and viscosity changes in the egg endoplasm which inhibit internal phenomena.

The writer wishes to express his gratitude to Dr. A. C. Giese and Mr. E. W. Lowrance, School of Biological Sciences, Stanford University, and Professor

Frank Coleman, Department of Physics, Howard University, for technical advice and assistance. He is also indebted to Dr. W. K. Fisher and the staff at Hopkins Marine Station for their many courtesies.

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THE ELECTRICAL CHARGE ON NUCLEAR CONSTITUENTS (SALIVARY GLAND CELLS OF SCIARA COPROPHILA)

LEON CHURNEY AND HERBERT M. KLEIN[1]

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In spite of the importance of electric charge in the behavior of colloidal materials, very little work has been done to determine either the sign or the magnitude of the charge on the particles of the cell nucleus. It is possible by cataphoretic experiments to determine at least the sign of the charge on the various structural elements of the nucleus. At the present time there is almost no direct data on the sign of the electric charge on animal chromosomes or on the nuclei of living animal cells. Botta is one of the few investigators who has attacked the problem. He reports that the chromosomes of embryonic chick heart cells are negatively charged during mitosis, but that there is little or no effect of the electric current on the chromatin during interkinesis. von Lehotzky states that he was unable to obtain results in his experiments on Protozoa and on frog blood cells.

It is possible to obtain definite information concerning the sign of the charge on giant chromosomes such as are found in the salivary glands of fly larvæ. The glands of the dipteran *Sciara coprophila* are especially favorable material for cataphoretic studies. The glands were dissected out of the larvae and studied in the Ringer's solution used by Bělař for grasshopper spermatocytes. The isotonicity of this solution with the salivary gland cells was determined by comparing glands in body fluid with those in the Ringer's solution.

The electrical set-up used in these studies consisted of a non-polarizable system of Cu–$CuSO_4$ electrodes with agar bridges. The lower extremities of two tubes, 6 mm. in diameter, were filled with a 3 per cent agar gel made up in Ringer's solution; above this gel was placed a saturated solution of $CuSO_4$, into which the ends of the copper wires carrying the current were introduced. A double-throw switch in the circuit permitted the reversal of the direction of current flow. A gland, dissected out in Ringer's solution, was placed in a drop of this medium on a glass slide, and a cover-glass was supported above it. The agar tips of the electrodes were placed in contact with the fluid, at opposite ends of the slide. By varying the amount of fluid between the electrodes the current strength was varied from approximately 5 to 25 milliamperes. The strength of the current passing through the nucleus was not, of course, determined.

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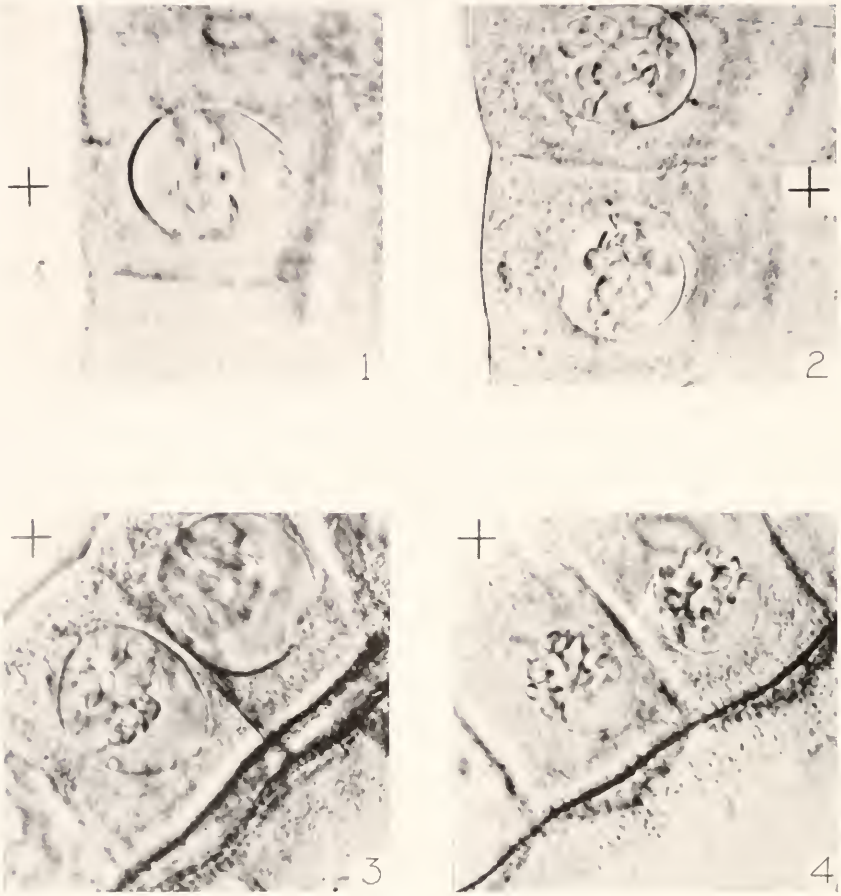
[1] We are pleased to acknowledge the aid of Dr. L. V. Heilbrunn of the University of Pennsylvania during the course of this investigation. We are also grateful to Dr. C. W. Metz of Johns Hopkins University for furnishing us with cultures of *Sciara coprophila*.

In the course of these experiments, one of the difficulties which presented itself was that of ascertaining whether or not the cell was alive. We believe that the nuclei and the chromosomes contained within them were alive, at least during their first responses to the passage of the current. In any case, all subsequent reactions were essentially the same, even in cases where there was reason to suspect that the cell was dead. After the current had passed through the cell for a short time, apparently irreversible cytosomal coagulation set in, indicating the death of the cell. In cases of extreme coagulation, very little, if any, response to the passage of the current was noted on the part of either the nuclei as a whole or the chromosomes.

In order that the chromosomes migrate cataphoretically, it is essential that the electric current pass through the gland cell and through the nucleus. Conditions must, therefore, favor such a passage of current. If the gland is placed in a large quantity of salt solution, practically all the electric current will pass around the cells. However, as the quantity of surrounding fluid, or its conductance, is decreased, the current will begin to flow through the cells. In the case of the salivary glands, the position of the gland in relation to the direction of current flow is of primary importance. If the long, spindle-shaped gland is parallel to the lines of current flow, the current will tend to flow along the central canal of the gland. On the other hand, if the gland is perpendicular to the lines of current flow, there is much more tendency for the current to pass through the cells, partly because of the fact that in this position the canal does not act as a short circuit, but also because of the fact that the path of the current leads through only two cells, and not through a long line of them.

The first apparent effects of the current flow on the nucleus were usually an increase in the nuclear volume, and an increase in the refractivity of the chromosomes. The nucleus as a whole, i.e., the nuclear membrane, moved toward the cathode (positive charge), whereas the chromosomes contained within it moved toward the anode (negative charge). These two opposing movements are simultaneous (see Fig. 4). When the direction of current flow was reversed, both the nuclei and the chromosomes responded instantaneously to the change by a corresponding reversal of their migration (see Figs. 1, 2 and 3). This reversal of migration was obtainable several times before the death of the cell and the subsequent coagulation prevented any response. When the circuit was broken, the nuclei and the chromosomes tended to return to



EXPLANATION OF PLATE I

Photomicrographs of unfixed and unstained cells of the salivary glands of *Sciara coprophila* larvæ, showing the effect of the passage of electrical currents. The anode is indicated, in each case, by a + sign. Figures 1, 2, and 3 are the same cells under different experimental conditions. Figure 4 shows cells of another preparation.

Fig. 1. Chromosomes migrating toward the anode, nuclear membrane toward the cathode. Note the crenation of the nuclear membrane as it is pressed against the cytosomal granules. Eight milliamperes.

Fig. 2. Same, direction of the current reversed. Chromosomes migrating toward anode. Eight milliamperes.

Fig. 3. Same, direction of current again reversed, establishing same polarity as in 1. Eight milliamperes.

Fig. 4. Another preparation. The nucleus as a whole is moving toward the cathode; its former position in the cytosome is indicated by the clear area toward the anode. The chromosomes are shown oriented toward the anode. Ten milliamperes.

their original positions in the cell. This was due, presumably, to a polarizing current in the reverse direction.

The movement toward the cathode and the simultaneous movement of the chromosomes toward the anode brought about distortions and abnormalities of the nuclear membrane (see Fig. 1). The chromosomes, in their movement toward the positive pole, often exerted sufficient pressure against the nuclear membrane to bring about an elongation of the nucleus in the direction of the current flow. On the other hand, the opposing force with which the nucleus as a whole moved toward the cathode was at times great enough to carry the chromosomes with it toward this pole (see Fig. 4). In such cases, however, the chromosomes always remained on the anodal side of the nucleus.

The nuclei, in their movement toward the cathode, tended to resume their spherical shape until the opposing motion of the chromosomes made this impossible, as described above, or until their movement toward the cathode was prevented by the densely packed granules of the cytosome. In the latter case, the nuclei often flattened themselves against these granules, while the anodal side of the nuclear membrane continued to flow inward (toward the cathode), carrying the chromosomes with it. This pressure of the membrane against the chromosomes often brought about a crenation of the membrane. Upon the breaking of the circuit, the nuclei tended to return to their former positions in the cells, and in doing so, tended to resume their spherical shape.

In cells which were mechanically crushed, nuclei often remained intact and floated partially or entirely free of the cytoplasm. Nuclei which were altogether free to move through the medium migrated toward the cathode. Since these nuclei were approximately midway between the slide and the cover-glass, their migration to the negative pole could not be due to the water current of endosmosis that flows in the opposite direction through the center of the chamber. The chromosomes, in these cases, were attracted to the anodal side of the nuclei, but with a force generally insufficient to distort the nuclei or to deter their rapid motion toward the cathode. Nuclei partially free from the cytoplasm, but adhering to the latter, displayed the characteristic reactions described in preceding paragraphs.

The observations indicate that the charge on the chromosomes is negative. This is in accord with various studies on plant chromatin (Pentemalli, McClendon, Hardy, Meier, Zeidler, von Lehotzky). Most of the observations of these investigators were made on material fixed after the exposure of the material to electric current, and such a procedure is perhaps open to question. However, von Lehotzky studied living plant cells.

The observation that the nucleus moves toward the cathode is new.

Perhaps other observers failed to note such a movement because of the fact that for the most part their observations were made on material fixed presumably after the cessation of flow of the current. In our own studies we observed that as soon as the circuit was broken the nuclei immediately tended to return to their original positions.

The positive charge on the nucleus as a whole is perhaps conditioned by a positive charge on the cytoplasmic colloids. Just as a quartz particle or a blood cell takes its charge from the colloids in which it is immersed and which are adsorbed on its surface, so the nucleus may owe its positive charge to the charge of protoplasmic colloids (in this connection compare Heilbrunn, 1928). The fact that isolated nuclei are positively charged does not necessarily invalidate this concept.

These experiments give us an indication of the complex electrical character of the cell components, in contrast to the excessive simplicity revealed in the cataphoresis of quartz particles or other inanimate systems. We have a living cell with a negative charge at the surface, perhaps a positive charge in the cytosome, a positive charge on the nucleus imbedded in it, and a negative charge within the nucleus (on the chromosomes).

In conclusion, this paper has attempted to stress the fact that the nucleus as a whole is positively charged, while the chromosomes within it are negatively charged. The complexity of the living system with respect to the electrical charge of the cell components has been contrasted with the apparent simplicity of inanimate systems. A more quantitative study of these phenomena is planned.

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DEVELOPMENT OF THE PRIMARY GONAD AND SEXUAL PHASES IN VENUS MERCENARIA LINNÆUS [1]

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INTRODUCTION

Sexuality of Pelecypoda has been a subject of scientific study since the end of the seventeenth century. A review of the literature on this subject reveals that in some of the forms that have been carefully studied many individuals exhibit at least one change of functional sexuality during their lifetime (Roughley, 1933; Coe, 1936*a*, 1936*b*). As a rule, a strong tendency toward protandry has been observed. Thus far, however, only two genera of Pelecypoda, namely, *Ostrea* and *Teredo,* have been extensively studied. It is true that these studies have contributed greatly to our knowledge of sex ratios and sexual phases in these bivalves, but it also seems possible that a thorough study of a representative species belonging to a genus widely differing in its mode of living from oysters or *Teredo* might furnish additional information leading to a better understanding of the sexual phenomena of mollusks. *Venus mercenaria,* the hard-shelled clam of the Altantic Coast commonly known as "quohog," was selected for this study.

This proved to be a fortunate choice, for the species was found to pass through phases of sexuality somewhat differing from those previously described for any member of the group. Like the teredos and the oysters, the hard-shelled clam shows a marked degree of protandry but there is no evidence of a second change of sexuality. The primary gonad is more or less distinctly bisexual but in nearly all individuals it becomes differentiated into a spermary when the animal is only a few millimeters in length and only a few months of age. The young individual may function as a male at the end of its first summer, retaining this phase of sexuality during the winter and becoming fully functional at the age of about one year. Others first become functional in their second summer.

About half of all the young clams examined retained the male phase of sexuality after the discharge of the spermatozoa, while an approximately equal number transformed to females. The sex ratios during the second winter and thereafter are thus equalized.

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[1] Part of the dissertation presented to the faculty of the Graduate School of Yale University in candidacy for the degree of Doctor of Philosophy.

[2] Published by permission of the U. S. Commissioner of Fisheries.

The writer desires to express his appreciation to Professor W. R. Coe for his advice and criticism throughout the course of this work.

Material and Methods

V. mercenaria is found along the Atlantic Coast from the Gulf of St. Lawrence to the coast of Texas. In the past few decades this species has become established on the Pacific Coast where it was transplanted with the eastern oyster.

The material generally used in this work was collected in Long Island Sound, near Milford, Connecticut. Young clams were obtained at regular intervals from a single clam bed and prepared for microscopic examination. A large number of small clams was also taken from concrete tide-filling tanks with a capacity of several thousand gallons, at the U. S. Fisheries Biological Laboratory in Milford, Connecticut, where environmental conditions, such as temperature and salinity of the water were observed and recorded. In the preparation of the material for study, standard methods of cytology and histology were employed.

In the present study all the observations on living material were checked by histologically prepared material in order to determine accurately the characteristics of the constituent cells of the gonads. The method employed by Spärck (1925), which consisted of boring a small hole through the shell of an animal and removing a piece of the gonad for examination, is not satisfactory, as very misleading conclusions may be reached by assuming the condition of the entire reproductive system from the examination of a single small sample of the gonad. Similarly, the method of Orton (1927), consisting largely of the examination of the living tissue to note the condition of the gonad with regard to the production of sex elements, is also open to criticism because of the impossibility of distinguishing many important details under such conditions.

Age and Size

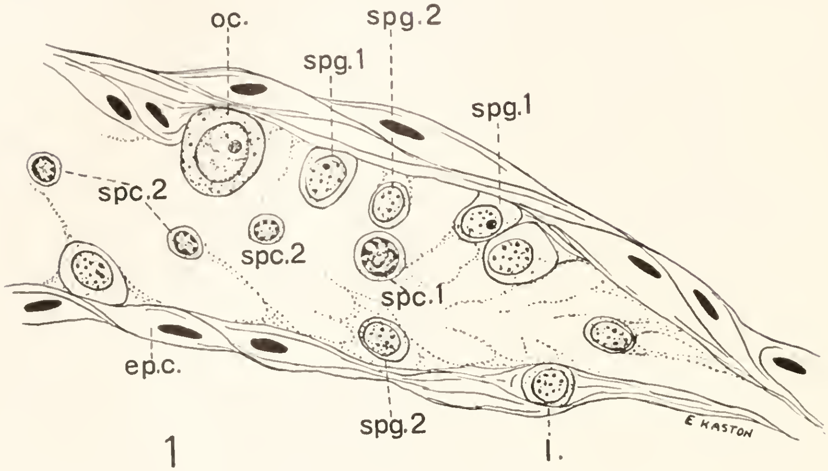
A review of the literature fails to disclose any important contributions on the subject of sexuality of *V. mercenaria* in general and on the development of the primary gonads in particular. The only work is that of Belding (1912), where he states that the average hard-shelled clam is capable of spawning when in its third summer, for sexual products could not be found at an earlier age. The sexes were found to be separate, each animal presumably remaining either male or female all its life. He gives no histological data, and it is apparent from his work that his conclusions are based only on macroscopic examination of living clams and upon his observations of their spawning activities.

It was impossible to obtain for this study small clams whose shell-length was less than 4 mm. long. Therefore the smallest animals studied were 4–5 mm. long. These were found at Trumbull Beach, Long Island Sound, during September and October. As the age of studied animals is of importance, several attempts were made to secure clams of a known age. This was accomplished by collecting seed clams and by keeping them under observation. There are two possible conclusions as to the age of small, 5–7 mm. clams, collected for this study during October–December; namely, that they set in the year they were collected, in which case their age would have been only a few months, or that they were about 14–18 months old, setting the preceding year. Belding (1912), studying *V. mercenaria* along the coast of Massachusetts over a period of five years, found that the rate of growth of the clam is largely determined by its environment, and that, as a rule, the growth in any bed is fairly uniform. In his experiments at Monomoy Point, he found that the average size of a 14-months-old clam (collected in October) was 25.59 mm. Judging by the description of the Monomoy Point experiment, the environmental conditions there closely resemble those of Trumbull Beach, at which place the young clams were obtained for the present work. The similarity of conditions of the two places makes it logical to assume that the rate of growth of clams at Trumbull Beach is more or less identical to that of clams of Monomoy Point. Thus, clams 5–7 mm. long, collected in October–December, presumably were of that, and not of the preceding year set. This conclusion is supported by the observation of Belding (1912) that young clams immediately after setting showed an average gain of 3.4 mm. per month, which would make a length of about 10 mm. by the end of November.

In the present paper all the remarks concerning the size of animals refer to the length of the shell.

Development of Primary Bisexual Gonad

Gonad tissue first appearing in the young animal consists of a very thin layer of cells between the muscular body wall and the stomach. Gonads of the juvenile phase first appear, not near the muscular body walls, but a short distance from them at the level of the heart or slightly below it. The follicles of the juvenile gonad are at first composed of a single layer of germinal epithelium cells. These cells of elongated type are rather irregular in shape and size and possess very large, deeply-staining nuclei. During the early stages there is virtually no lumen



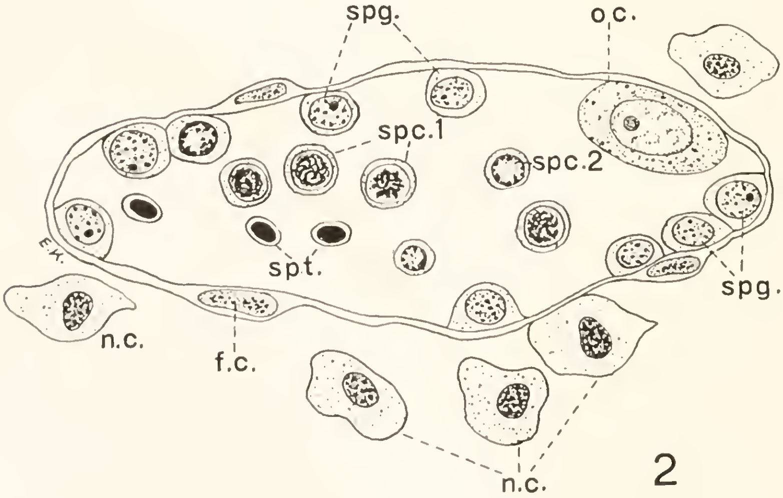


Fig. 1. Primitive bisexual gonad of a young clam soon after formation of the lumen. *I*, indifferent cell; *ep.c.*, follicular cells; *oc.*, young ovocyte; *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes.

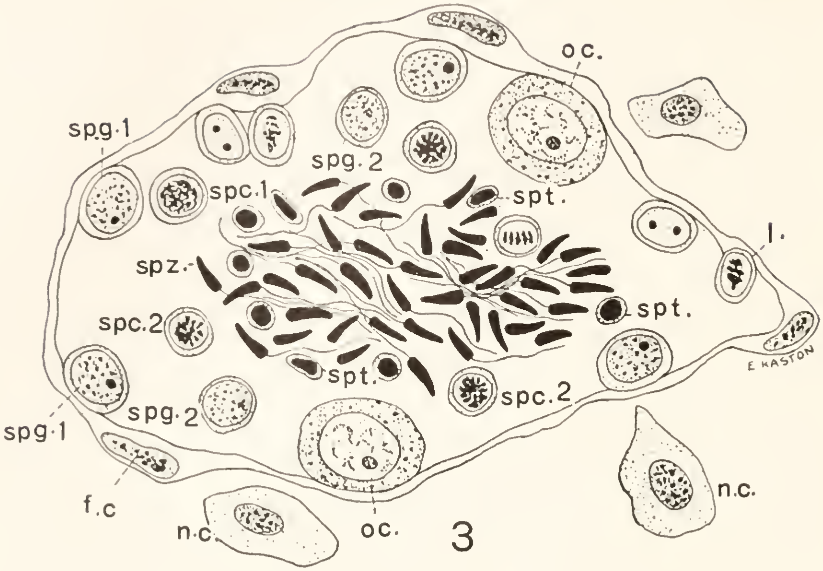
Fig. 2. Primary bisexual gonad in a young clam (4-6 mm.) showing large ovocyte (*oc.*), spermatogonia (*spg.*), primary spermatocytes (*spc.1*), secondary spermatocytes (*spc.2*), and spermatids (*spt.*); *f.c.*, follicular cells. Nutritive cells (*n.c.*) are seen near the follicular wall.

formed, the walls of the follicles being almost in contact with each other. As the animal grows, the follicles of the primary gonad begin to ramify through the loose connective tissue. A few weeks later the germinal epithelium begins to differentiate into gonia and at this time the lumen in the follicle is formed (Fig. 1). Rapid proliferation and specialization of cells follows.

In small animals, 5–12 mm. long, the gonad does not extend very far in the ventral direction, seldom reaching below the line corresponding to the middle part of the stomach. The follicles of the gonad are not very numerous, and usually only six to eight of them can be found in one cross-section of the entire animal. In cross-sections the gonad follicles of such animals already show the lumen, which is usually oval or round in shape with a greatest diameter of 50–100 microns. Careful study of sections reveals that different gonad follicles of the same animal exhibit a widely different degree of development and bisexuality. Some of the follicles may consist of only a few indifferent cells while in others the germinal epithelium has already differentiated into male and female cells. All degrees of such processes can be observed. In some cases the follicle contains a few gonia along its wall and a mass of spermatogenic cells in early stages of development in the lumen (Fig. 2). In other, more advanced, cases spermatozoa are already formed, sometimes occupying the largest part of the lumen, while a few small ovocytes showing mitochondrial filaments of yolk nuclei are found along the walls of the follicle (Fig. 3). Gonads at such stages are distinctly bisexual and because the proliferation of spermatogenic cells is very rapid such young gonads acquire a strong male appearance (Loosanoff, 1936*a*).

In some cases the appearance of the follicles looks as though the spermatozoa were already discharged. For instance, a large sample of animals 5–7 mm. long was collected on Trumbull Beach in October, 1934. Upon examination of the prepared material it was observed that some of the follicles had already discharged spermatozoa. The lumen of such follicles was large and empty while along its walls young ovocytes and indifferent cells were present. As a rule, few phagocytes could be found inside the lumen (Fig. 4). With the purpose in view of establishing the age of those animals, very careful examination of their shells was made under a dissecting microscope. In not a single case was an indication of the winter ring found. The outer surfaces showed an embryonic shell and 7–13 growth lines formed at approximately equal intervals (Fig. 5). Thus the studies of the shells indicated that the animals of that sample were only a few months old.

If the evidence that *V. mercenaria* produces and discharges gametes during the first summer of its life is reliable, then it resembles in this respect many of the other bivalves whose sexual development is well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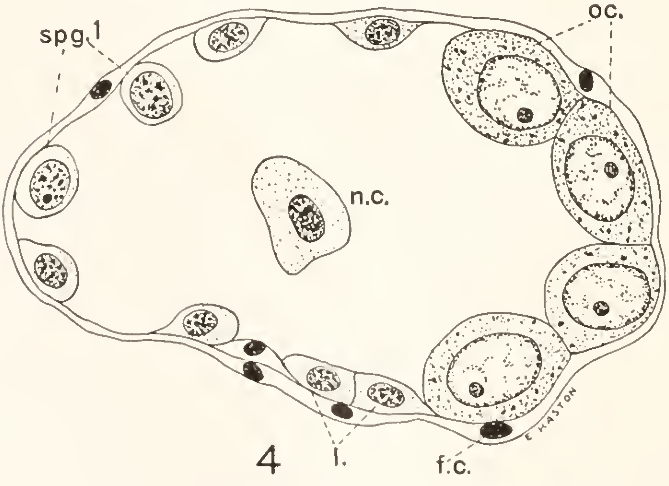
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FIG. 3. Primary bisexual gonad of predominantly male character. Spermatozoa are present, occupying the center of the lumen. A few ovocytes (*oc.*) are lying along the follicular wall. *I*, indifferent cells; *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids in different stages of spermioteleosis; *spz.*, spermatozoa; *n.c.*, nutritive cells; *f.c.*, follicle cells. Size 7–12 mm.

FIG. 4. Gonad follicle of a young clam (7 mm.) after discharge of the first crop of spermatozoa. *I*, indifferent cells; *oc.*, young ovocytes; *spg.1*, primary spermatogonia; *n.c.*, phagocyte; *f.c.*, follicle cells.

known (Nelson, 1922; Coe, 1936*c*). It is apparent, however, that there cannot be a definite rule applied to all Pelecypoda mollusks as to the time when they first form functional gametes. As a matter of fact, considerable differences are often found in closely related species and in different localities, as Spärck (1925) has so clearly demonstrated in *O. edulis*. *O. lurida* is capable of passing through three sexual phases during the first year of development in warm waters (Coe, 1932), while *O. virginica* of the northern part of the eastern coast of the United States does not form any ripe gametes until the second year (Needler, 1932). In *V. mercenaria*, ripe spermatozoa are formed within 3–5 months after setting. Among several hundred young animals studied, approximately 98 per cent of them passed through such a protandric male phase. The remaining 2 per cent appeared to develop into females without passing through a functional male phase.

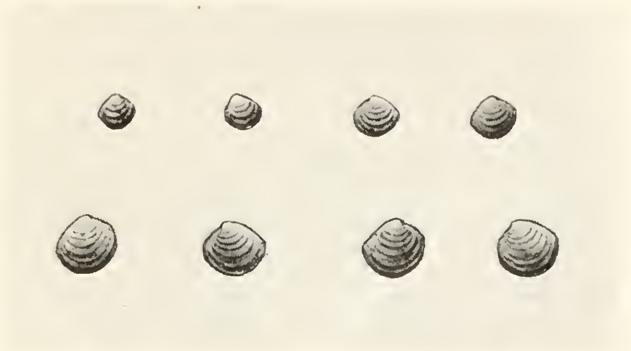


Fig. 5. Shells of the young clams, age about 3 to 4 months, collected in October–November, showing the absence of the winter ring. All of these were already provided with gonads containing mature spermatozoa. Natural size.

Condition during First Winter

According to the writer's observations, the shell-growth of clams in Long Island Sound ceases in November. Small (6–10 mm.) clams examined at that time differ very little from those studied in October. Again gonad follicles in various stages of development are found. In many follicles spermatogenesis continues and cells of all stages of development, including spermatozoa, are present (Fig. 6). In other cases spermatozoa have already been discharged and the distended lumen is virtually empty (Fig. 4). Often numerous phagocytes are seen invading the lumen or attached to the walls of the follicle. Those presumably ingest the residual spermatic cells. In a few instances the gonad tissue has already ramified ventrally below the stomach and individual follicles can be seen confined to the space between the muscular body wall and the digestive gland.

By the middle part of December the temperature of the water of Long Island Sound reaches the point at which the period of hibernation for this species begins (Loosanoff, 1936*b*). Examination of young animals collected during that period, which extends until the middle part of April, or until the water temperature rises above 5.0° C., reveals that changes of only minor importance occur in the primary gonads. There is apparent neither increase in the number of cells constituting the gonad, nor increase in the size of the follicles. Spermatogenesis is virtually discontinued and cells of the intermediate stages are few in number. The spermatozoa, however, are retained in large numbers in many of the follicles. Unless carefully examined, many of the animals at this stage may be mistaken for true males, but the presence of young ovocytes showing distinct mitochondrial bodies and yolk nuclei indicates the actual

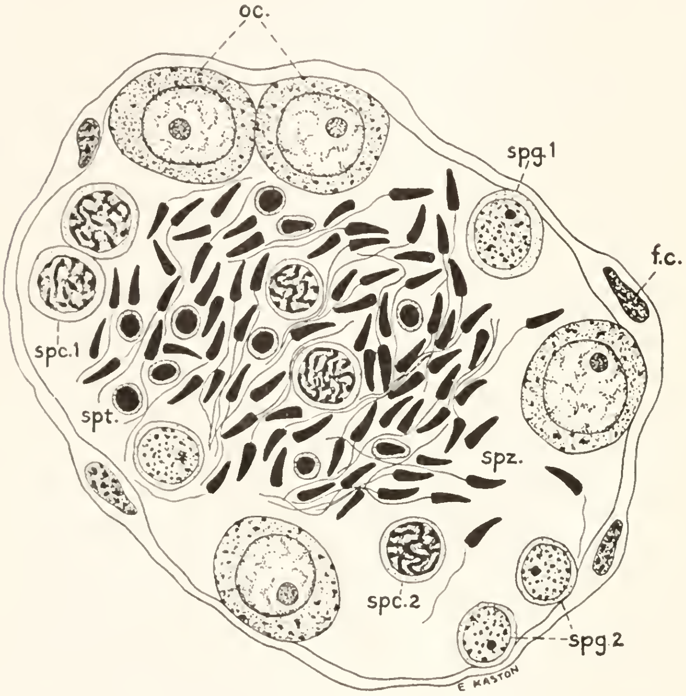


FIG. 6. Bisexual primary gonad of a young individual about 16 weeks of age showing the cells of both sexes but predominantly of male type. *spg.1*, primary spermatogonia; *spg.2*, secondary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei; *f.c.*, follicle cells.

bisexual character of the gonads. In other cases the bisexuality of an individual is more easily noticed because of the large number of young ovocytes present (Fig. 6). A few pycnotic cells are sometimes seen.

Hibernation continues until the middle of April. The lowest temperature of the year is reached in February or the first part of March. After the middle of March the water temperature begins to rise very slowly and reaches 5.0° C. by the middle of April.

Condition in Spring and Summer

By the time the water temperature reaches 7.0–8.0° C., which usually takes place at the end of April, some minor changes in the gonads of young animals begin to occur. They are manifested by resumption of spermatogenesis in some of the individuals. The process, at that time, is very slow, and newly-formed cells, mostly spermatocytes of the first and second order, are few in number. The majority of animals still have gonads in the state resembling that of the hibernation period. As soon as the water temperature advances to a 10° C. mark, usually by the middle of May, more pronounced changes take place in the gonads. Active spermatogenesis is resumed in the follicles of many animals. Numerous gonia, primary and secondary spermatocytes are formed but few spermatids are seen. In many follicles spermatozoa retained since the preceding autumn are present. At this time a slight extension of the follicle is already noted. Phagocytic-nutritive cells begin to surround the follicles in which gametogenic activities are going on. Gonads continue to retain their bisexual nature because many small ovocytes measuring 20–30 microns can always be found in all follicles, even those showing a distinct preponderance of spermatogenic cells. Young ovocytes are, as a rule, found lying in contact with the follicular wall. Their large nuclei make them easily distinguishable from other cells.

Phagocytic-nutritive cells are often found in large numbers along the outer walls of the gonad follicles and a few penetrate into their lumens (Fig. 7). These cells, measuring up to about 12–14 microns and having a nucleus 4–4.5 microns in diameter, occur as a rule near the follicular wall. Their numbers appear to vary with the seasons, the greatest number being found during the active stages of gametogenesis. They are found during all stages of development from indifferent gonad to mature stages. Often some of them project through the follicular walls into the lumen, while others invade it. In clams such cells appear to perform functions of two types. First, their intimate contact with the gonad during the most active stages of gametogenesis indicates that they contribute certain substances necessary for the developing cells. Thus, their nourishing function may be assumed. Second, their presence inside the gonad follicles immediately after spawning suggests their purely phagocytic rôle of removing partially cytolysed, degenerated and residual cells.

As the season advances and the water temperature gradually increases, reaching about 15.0° C. by the end of June, rapid proliferation of sex cells progresses in a parallel manner. Gonads of young animals examined at that time of the year show that spermatogenesis proceeds very actively and that spermatogenetic cells in all stages of development are filling the follicles, often occupying the entire space of the lumen. It often happens that various follicles of the same animal show quite different stages of development. Frequently one of the follicles possesses a large number of spermatozoa, while another follicle, situated next to it, has most of its cells in early stages of spermatogenesis. Nutritive cells are quite numerous, sometimes entirely surrounding the follicle (Fig. 7). The clams are feeding and growing rapidly, many of them showing an increase in total length of shell of 3 mm. since the end of the hibernation period.

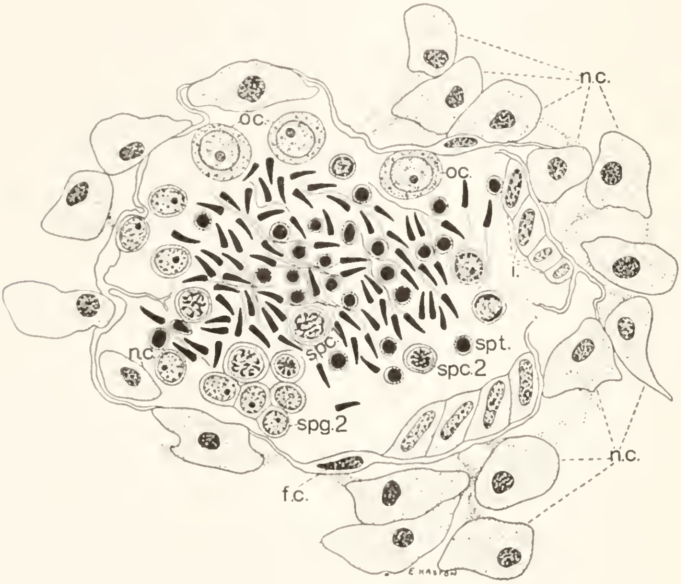


Fig. 7. Gonad of the young clam (1.0 cm.) surrounded by the nutritive phagocytic cells (*n.c.*). Other letters as in Fig. 6. Late April–May.

On examining the clams passing through active gametogenesis, one's attention is immediately drawn to the large numbers of nutritive cells surrounding the outer walls of the follicles. In the follicles themselves great activities are noticed. They are quite distended and filled with spermatogenetic cells. At this time mature spermatozoa are already more numerous than cells of other stages. Spermatocytes in all stages of development and spermatids in spermioteleosis are found. Regardless of such spermatogenetic activities and the distinctly male character of gonads, young ovocytes are always found in small numbers along the wall of the follicles. There is a rapid extension of the branching system of follicles, but this extension is directed chiefly in the posterior direction and not ventrally; consequently very few follicles extend below the level of the stomach.

As has been mentioned before, some of the animals examined during the winter had follicles virtually devoid of all except indifferent cells. Such animals apparently discharged their spermatozoa in the fall. In June and July those animals also exhibit gametogenetic activities and spermatozoa are rapidly formed. It is very interesting to note, however, that in such cases the follicles usually contain more female cells than the follicles of the animals which retained spermatozoa throughout the winter. The variation in the proportion of cells characteristic of the opposite sexes found in clams of the same age strongly indicates that genetic factors and probably the effect of environment influence the production of sex cells.

Spawning

In August the water over the natural clam beds reaches a temperature high enough to induce the spawning of clams. If young clams, which by this time have reached the size of 2.0–3.0 cm. are examined, their gonads reveal the fact that the spawning process is not a phenomenon of short duration but lasts for some time. Different follicles of the gonad of the same animal contain sex cells in various quantities. Some follicles have already discharged their contents while in other follicles spermatogenesis is in progress and the lumen is still filled with mature spermatozoa. Apparently, several days, or perhaps weeks, are required for young clams to complete their spawning. Furthermore, by examining a sample consisting of many animals, the conclusion can be reached that there is considerable difference in the spawning behavior of individuals because some of the animals collected at the same time and from the same place have their sex products completely discharged, while others still retain their spawn in various quantities. This indicates that the entire population of a certain bed does not begin spawning at exactly the same time, and that the spawning season of clams extends for a considerable period of time.

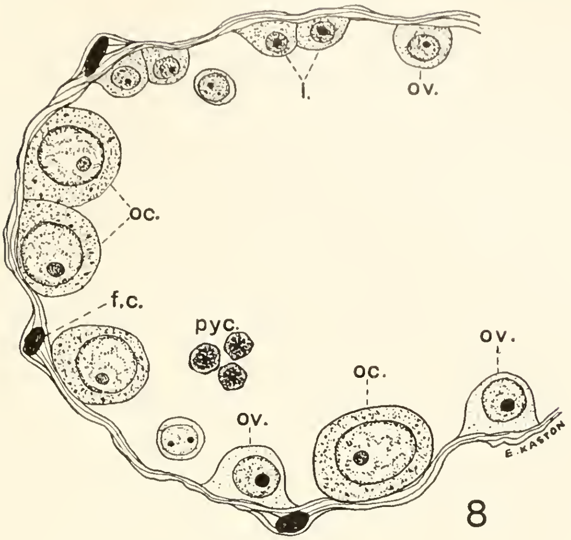
In many follicles, which during the spawning period are partly or fully freed of spermatogenic cells, young ovocytes, many of them in the spireme stage, are growing along the walls. In some instances groups of ovocytes occupy considerable portions of follicles. A few nutritive cells may be observed in the vicinity of follicles whose contents are already discharged. Branching gonad tubules begin at this time to extend ventrally, occupying the space between the body walls and the digestive gland.

During the months of August and September the animals grow very rapidly, reaching the size of 2.5 to 3.2 cm. Many animals have grown as much as 2.0 cm. since the end of the hibernation period. Simultaneously with the growth of the animals, ramification of gonad follicles proceeds. The follicles spread in all directions and envelop the stomach and intestines as well as the spaces in connective tissue between the body wall and the digestive gland.

Transformation to Definitive Males and Females

In September, after the spermatozoa have been discharged, two types of individuals become distinguishable as definitive males and females. In the males a second or third period of spermatogenesis begins in the autumn and continues at a reduced rate throughout the winter. Mature spermatozoa are retained in the follicles all this time. Many young ovocytes and numerous indifferent cells are also present. In the spring, with the increase of water temperature, renewed rapid branching of follicles takes place and the gonads then begin to acquire the typical male character of the adult. Spawning follows later in the summer.

In animals destined to become females radical changes take place in the gonads upon the completion of the initial male phase. After the discharge of spermatozoa the follicles are left in a distended state. Their lumens are large and empty (Fig. 8) with the exception of a few pycnotic bodies consisting of cytolysed spermatogenic cells. An irregular layer of indifferent cells, ovogonia and primitive ovocytes measuring up to 20–25 microns, and a few spermatogonia remain for a time along the walls of the follicles. Nutritive cells lie in close contact with the gonad walls and occasionally can be found inside the lumens of the follicles. No significant changes occur in the gonads of future females during the hibernation period, for during all this time the follicles remain distended, round or oval in outline, with virtually empty lumens. There is little indication of ovogenetic activities. In the middle of May, regardless of the fact that the water temperature is 9.7° C. (i.e., about four degrees above hibernation mark), and that the animals already had been feeding for approximately one month, their gonads remain in a semidormant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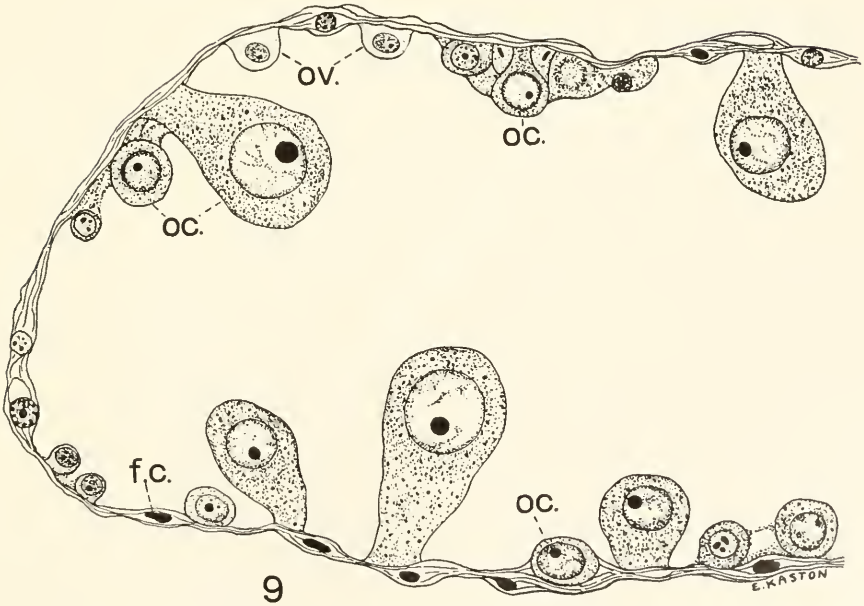
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Fig. 8. The gonad of the animal destined to become a female after the completion of the initial male phase. *I*, indifferent cells; *ov.*, ovogonia; *oc.*, young ovocytes; *pyc.*, pycnotic cells; *f.c.*, follicle cells.

Fig. 9. Early ovogenetic activities in the young female clam producing its first crop of eggs; *ov.*, ovogonia; *oc.*, ovocytes in various stages of development; *f.c.*, follicle cells. Age 18–19 months. Middle of May.

state. Only in rare instances are synaptic activities in young ovocytes noticed. In some follicles, however, pycnosis and phagocytosis of spermatogenetic cells left over from the last autumn proceed very rapidly.

In clams producing their first crop of ova, slow ovogenetic activities become apparent in the middle of May and active ovogenesis begins in June when the water temperature approaches 15° C. Within a few days after such a temperature has been reached the appearance of the gonads undergoes a marked change. Young ova in various stages of development, some of them 42–45 microns in size, grow from the walls of the follicles into the lumen (Fig. 9). The growth and proliferation of ova are very rapid and the animals formerly functioning as males have now reached the stage of functional females. Not all the follicles begin to produce ova at the same time. Frequently, in the same animal some of the follicles contain large, rapidly growing ova, while a few others are still in an apparently dormant state showing many indifferent cells along their walls, ovogonia and very minute ovocytes.

The first crop of eggs produced by the young female is usually small and in this respect they can always be distinguished from fully adult individuals, because in the latter case the follicles contain many more ova. After spawning, the gonads of both sexes may contain some cells characteristic of the opposite sex, thus retaining their bisexual character. It is especially noticeable in the case of males, where comparatively large ovocytes are easily distinguished from male cells.

FUNCTIONAL HERMAPHRODITISM

Pelseneer (1926) has shown that hermaphroditism is quite common among mollusks. Among Pelecypoda numerous cases of partial and true hermaphroditism have been reported. As has been stated in the chapter on the development of the primary gonads of *V. mercenaria*, many grades of bisexuality occur in that animal during the first two years of its life. The examination of the gonads of adult males also reveals, in almost every instance, the presence of small ovocytes somewhere along the walls of the follicles. In all such cases, since the cells of both sexes are present in the gonads of the same individual, the term "partial hermaphroditism" may be applied. However, under functional or true

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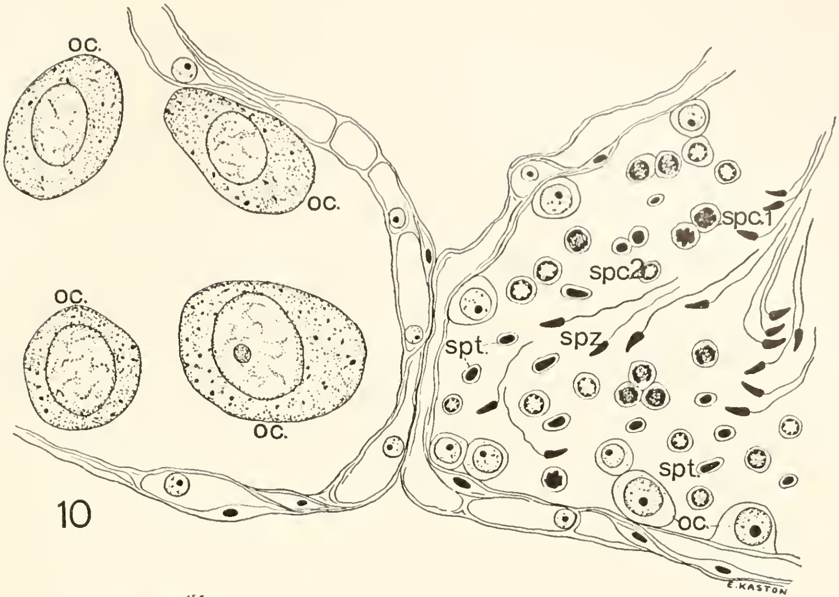
FIG. 10. Functional hermaphroditism in *Venus*. Portions of two adjacent follicles of the same individual containing cells of opposite sexes. *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei.

FIG. 11. Portion of hermaphroditic gonad showing the ripe ova surrounded by spermatogenetic cells. *spg.1*, primary spermatogonia; *spc.1*, primary spermatocytes; *spc.2*, secondary spermatocytes; *spt.*, spermatids; *spz.*, spermatozoa; *oc.*, large ovocytes showing mitochondrial bodies and yolk nuclei.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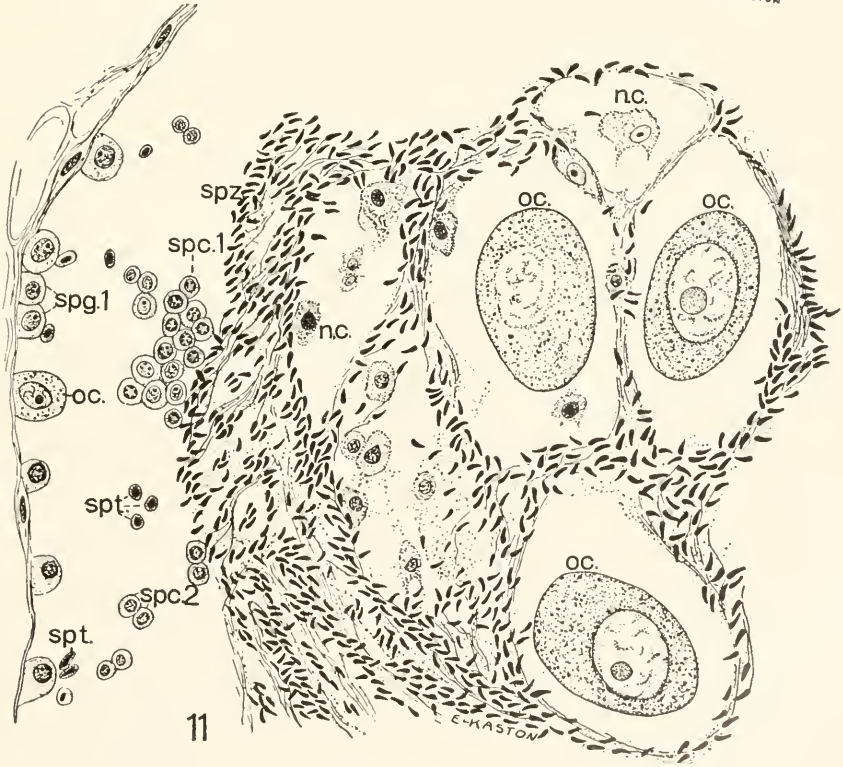
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FIGURES 10 AND 11

hermaphrodites, only those animals should be included whose gonads possess masses of cells of both sexes, in which case self-fertilization may be possible.

In *V. mercenaria* the adults are, with few exceptions, of separate sexes. Among 650 mature clams studied by means of serial section, only 3 cases of functional or true hermaphroditism were observed. In one case fully ripe ova were found in some of the follicles, while spermatozoa occupied an adjacent but separate portion of the gonads (Fig. 10). In two other cases a few ripe ova were occupying the lumen of the follicle in which spermatogenetic activities were also almost completed. Numerous ripe spermatozoa surrounded the ova lying in the follicles (Fig. 11). In all of these instances the ova and spermatozoa may be discharged simultaneously into the mantle cavity of the animal aud then into the water where abundant opportunity for self-fertilization offers itself.

SUMMARY

1. Examination of the developing gonads of young clams from Long Island Sound, at two-week intervals during the first year of life, shows that a primary bisexual gonad is formed in each individual when the animal reaches the size of 4–6 mm.

2. The primary gonads form from associations of germinal cells in the connective tissue, not immediately beneath the body walls but at some distance from them, at the level of the heart or slightly below it. A lumen soon appears in each gonad follicle and the germinal cells begin to proliferate rapidly.

3. The primary gonad contains the antecedent cells of both sexes but the protandric nature of the primary gonad becomes manifested by the rapid proliferation of the spermatogenic cells.

4. This species is not strictly protandric because few individuals develop into females without passing through a functional male phase.

5. Functional spermatozoa have been observed in the gonads of young clams, collected in October and November when only 5–7 mm. long. In some cases discharge of spermatozoa occurs at that time.

6. In September of the second year, after the animals have spawned as males, two types of individuals become distinguishable as definitive males and females. In the males a second period of spermatogenesis begins in the autumn and continues at a reduced rate throughout the winter. The bisexual character of the definite sexual gland is usually retained to at least some extent because of the presence of many small ovocytes.

7. In animals destined to become females, after the completion of the initial male phase, the gonads may remain virtually empty but in a distended state throughout the winter. There is little indication of ovogenic activities until the following spring when the water temperature approaches 15° C. From then on the proliferation and growth of ova proceeds very rapidly until the animal reaches the functional female phase.

8. In *V. mercenaria* the adults are, with few exceptions, of separate sexes. Among six hundred and fifty mature clams studied by means of serial sections only three cases of true hermaphroditism were observed.

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SEASONAL GONADAL CHANGES OF ADULT CLAMS, VENUS MERCENARIA (L.)[1]

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INTRODUCTION

The literature on Pelecypoda mollusks contains but few accounts of systematic studies of the changes occurring in the gonads of an animal throughout the entire year. This phase of investigation of sexual phenomena in mollusks, though neglected so far, is, however, of considerable importance and interest because, if properly carried out, it could assist greatly in the understanding of the phenomena of change of sex in some forms or add to the knowledge of the alternation of sexual phases in others. Amemiya (1929) reported a very interesting case of sex change in the Japanese oyster, *O. gigas*, which was previously considered dioecious. His method consisted of making a hole in the shell of an oyster and removing through it a small piece of gonad for determination of sex. Following this the oysters were placed in cages and returned to the water for a period of one year, at the end of which they were re-examined. Meanwhile, no samples were taken for histological studies to determine what cellular changes precede the reversal of sex. Nelson (1928) gave a more or less detailed description of changes occurring in the gonads of the adult *O. virginica* from April until June, but did not continue his studies through the post-spawning time and season of hibernation. In other studies dealing with sexual phenomena of the genus *Ostrea*, a systematic description of changes occurring in the gonads of adult animals throughout the year is also lacking.

This paper deals with the changes occurring in the gonads of adult clams (*Venus mercenaria*) at different seasons of the annual cycle. The animals were studied for three successive years. Samples for this study were collected at bi-weekly intervals from the clam beds located near Charles Island, Long Island Sound. The depth of the water over the beds was 8–12 feet at mean low water.

Since the calendar's new year, occurring in the middle of winter, coincides with the prolonged period of the animal's inactivity, it is thought convenient in this study to consider the beginning of a new year from the ecological point of view, i.e., after the completion of spawning activities. It so happens that the spawning of *V. mercenaria* in Long Island Sound occurs at the time when the water temperature reaches its highest mark of the year. Ecologically this also represents the terminal point of the annual cycle. Therefore, immediate post-spawning time will be considered as the beginning of the ecological year, and the description of the changes occurring in the gonads of *V. mercenaria* will begin at that date. Sexes will be considered separately, the changes occurring in male gonads being described first.

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The writer desires to express his appreciation to Professor W. R. Coe for his advice and criticism throughout the course of this work.

MALES

The spawning of *V. mercenaria* is usually completed at the end of August or in the early part of September. The gonads of males examined during that period show large distended follicles containing but few sex cells. The center of the lumen is empty and the few spermatozoa that are still left remain near the walls of the follicles undergoing cytolysis. Spermatids and spermatocytes of both orders are virtually absent from all the follicles, but large primary gonia, a few secondary spermatogonia, indifferent cells and, occasionally, young ovocytes are commonly found lying in contact with the follicular walls. In many instances large, deeply-stained pycnotic cells, apparently the product of abnormal spermatogenetic activities, may be seen.

Male clams examined late in September and early in October reveal that the post-spawning period of recuperation has already passed, and the gonads of the animals are rapidly producing sexual cells for the next year. The gonad follicles at that time are occupied with spermatogenetic cells in various stages of development. Along the walls of the follicles numerous spermatogonia grow rapidly, often forming two or three rows of cells. Further in the lumen, occupying nearly all the available space, spermatocytes of both orders and spermatids in early stages of spermioteleosis can be seen. Fine protoplasmic strands connect the cells with the follicular walls. Undoubtedly the nutritive materials are delivered to the developing cells through these strands. In the majority of cases no ripe spermatozoa have yet been formed in the follicles. It is very characteristic that at this time, as well as at all other times of the active gametogenetic activities, the follicles are surrounded by a great number of nutritive-phagocytic cells. The water temperature over the clam bed at the time of collection of the samples was about 19.0° C.

By the middle of October the bottom water temperature of Long Island Sound decreases to about 16.0° C. Examination of the gonads at that time shows that spermatogenesis is continuing and that spermatozoa have already been formed in many of the follicles. As usual, numerous individual variations can be observed in the numbers of spermatozoa found in different follicles. Some of the follicles have the largest part of the lumen already occupied by apparently mature spermatozoa, while in others few cells in such an advanced stage can be seen. In general, the cells of the early stages of spermatogenesis still predominate. By the end of October (water temperature 12–13° C.) spermatozoa can be found in virtually all the follicles.

Regardless of the fact that during November the temperature of the water decreases rapidly, reaching about 8.0° C. at the end of the month, the spermatogenic activities of the clam proceed without interruption. By this time spermatozoa are by far the most numerous cells in the follicles. Spermatids in all stages of spermioteleosis also are present in large numbers, while spermatocytes and spermatogonia are much less numerous than in the animals examined in October and the early part of November. Many spermatocytes are seen in the synaptic stage or in the process of division. Practically the same condition exists in December. In all cases a large number of pycnotic cells are found scattered among the healthy ones and the phagocytic cells are actively engaged in clearing them from the follicles.

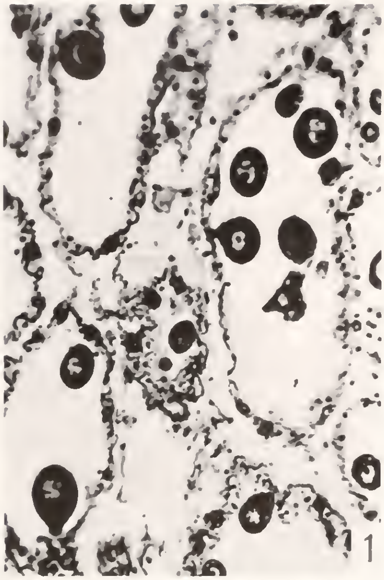
The middle part of December may be considered as the threshold of the winter hibernating period of *V. mercenaria* of Long Island Sound (Loosanoff, 1936*a*). The water temperature by that time decreases to a critical point of about 5.0° C. and the animal, confronted by an unfavorable environment, undergoes important physiological modifications which induce hibernation. But if the gonad sections prepared from animals collected during the early part of the hibernation period are compared with those collected one or two months previous to its onset, little difference can be noticed in their character. Throughout January and February spermatogenetic activities are continued at a reduced rate.

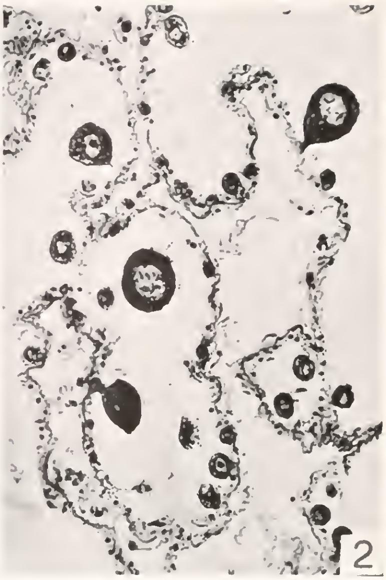
Animals collected in February possess large sex glands, enveloping the stomach, liver, intestine, and penetrating into the foot. The follicles are fully extended and filled with spermatogenetic cells of which spermatozoa are the most numerous. Excluding a brief post-spawning period, the spermaries of adult males of *V. mercenaria* contain morphologically mature spermatozoa at all seasons of the year. Spermatozoa, if removed from the spermaries and placed in a dish of sea water, after 2–3 minutes of quiescence begin to swim actively in their typical spiral way. This simple experiment was tried at bi-weekly intervals throughout the year and always, except in the short post-spawning period, gave positive results (Loosanoff, 1936*b*). The question of physiological ripeness of spermatozoa found in clams at all other times, except prespawning and spawning periods, still remains open because no eggs capable of fertilization could be obtained for such an experiment in late fall, winter and spring.

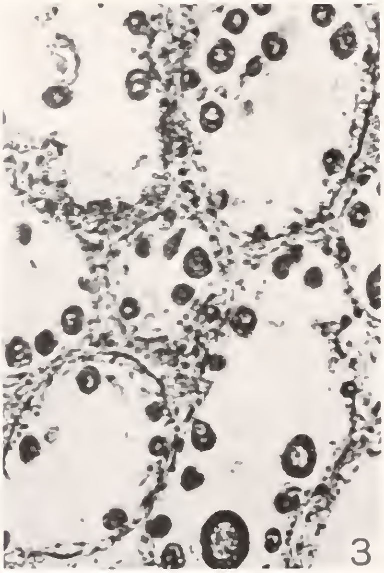
The month of March may be regarded as the time of the year when the spermatogenetic activities of the clam are the least conspicuous. The spermaries of animals collected during that month contain mainly spermatogonia and spermatozoa, while the cells of the intermediate stages are present only in small numbers. In the middle of April the water temperature passes above the critical hibernation point for clams. Nevertheless, as far as production of gametes is concerned, the animals do not respond noticeably to the favorable change in environment. The gonads remain quiescent, showing little spermatogenetic activity until the middle of May, when the water temperature begins to fluctuate between 10.0° and 13.5° C. As soon as the temperature reaches 15.0° C., which in Long Island Sound takes place early in June, vigorous spermatogenesis is again initiated and production of spermatozoa proceeds at an extremely rapid rate. The presence of a large number of spermatids in the follicles is very characteristic of this part of the year. Simultaneously with increase in water temperature further increase in activities of spermaries is noted. In June, July and during the first part of August many animals are found with ripe gonads. In several cases it was possible to induce such animals to spawn by a gradual increase of the water temperature. Among animals living in very shallow water or on tidal flats, individuals with partly discharged gonads may be found early in July, but the largest part of the clam population spawns in August when the curve of water temperature reaches its culmination point.

Females

During the immediate post-spawning period, after the gametes have been discharged, the sexes of *V. mercenaria* can still be distinguished easily. A description of the post-spawning condition observed in the clam spermaries has already been given. The ovaries in the post-spawning stage are characterized by the presence of a few unspawned, but apparently ripe, ova and by a large number of ovogonia and very young ovocytes. The presence and normal appearance of these ovocytes during the stage when sex reversal may occur in mollusks, points toward the conclusion that in *V. mercenaria* the change of sex from female to male seldom takes place.







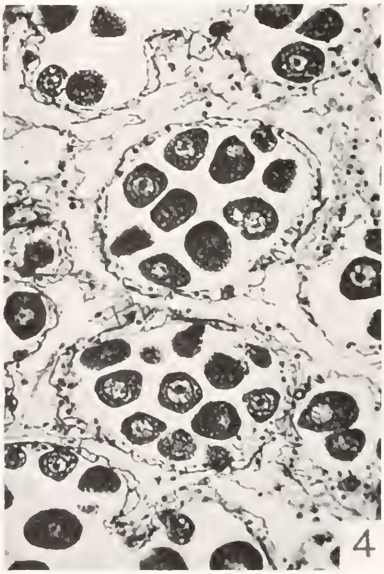


Fig. 1. Female gonad immediately after partial spawning. Follicles with unspawned ova are in a semi-contracted state. Early August. × 300.

Fig. 2. Female gonad in September. The ovarian follicles are still in a contracted state. Many young ovocytes are growing. A few unspawned ova still remain. × 300.

Fig. 3. Female gonad in October. × 300.

Fig. 4. Female gonad in November, showing the rapid growth of young ovocytes. × 300.

The number of large unspawned ova found in the ovaries of recently spawned clams varies greatly with individuals. In some cases almost the entire crop of ripe ova is discharged by the time spawning activities end, while in the majority of cases, a comparatively large number is retained. The difference between the follicles of the same animal, as to the number of unspawned ova left, is often very marked. Almost immediately after spawning the ovarian follicles begin to contract. This contraction does not proceed very far, however, and they are left in a semicontracted state with unspawned ova still retained in the lumen (Fig. 1). An almost continuous layer of ovogonia, indifferent cells, and young ovocytes in various stages of development and growth is often distinguished along the inner walls of the ovarian follicles.

The spawning of an individual clam is not completed in one attempt but is extended for a certain period of time, depending upon the individual and ecological peculiarities. Female clams with partly discharged sexual products were found on our experimental bed throughout August and September. Their ovaries contained, in various proportions, the cells of old and new crops. Towards the end of September only a few ripe ova were found. Apparently a delayed but nevertheless normal discharge of eggs takes place this late in the season.

The discharge of the ripe eggs evidently removes the factors inhibiting the production and growth of a new crop of ovocytes. As soon as the ovaries are freed of the bulk of their ripe eggs, ovogenetic activities are commenced. At first a few young ovocytes are seen growing and protruding towards the ovarian cavity (Fig. 2). The new ovocytes at this time vary markedly in size. The average size is approximately 17–20 microns but some of the youngest ones measure only 8–9 microns, and the largest, which are less numerous than others, reach the size of 35–40 microns. In almost every case studied the ovarian follicles were found to contain a few very large ovocytes measuring 55–60 microns. Such ovocytes, many of which were still connected by their egg stalks to the ovarian walls, represented the cells of the old crop.

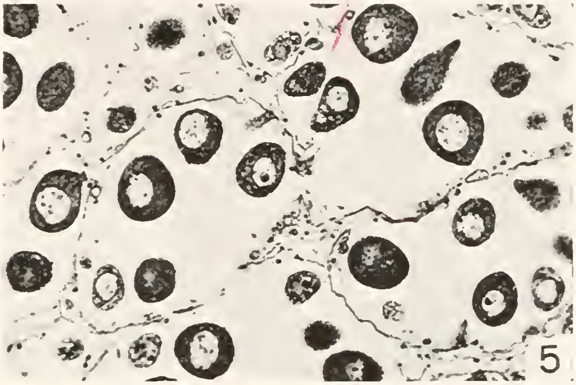
Early in October a considerable increase in the number of new ovocytes is observed. Many of these ovocytes are still of a rather small size as compared with fully grown eggs, a few of which are left in the ovaries (Fig. 3). At this time the average size of a new crop of ovocytes is about twenty-five microns. It should be remembered, however, that individual differences are great. It is often found that in a sample composed of a large number of animals a few individuals may have their gonads in either a greatly retarded or an advanced stage, as compared with the conditions observed in others of the same samples. This is sometimes very confusing and unless the sample studied is composed of a large number of animals, erroneous conclusions may be reached.

During the latter part of October and in November the growth of young ovocytes is very rapid. Some cells of the new crop have already reached the size of a mature egg but most of the cells are between 33 and 55 microns in size. There are still many minute ovocytes making their appearance but their number is considerably smaller than it was in the early part of October (Fig. 4). Simultaneously with the growth of the ovocytes, the follicles expand and often proliferation and formation of new follicles in the surrounding connective tissue can be observed. The young sex cells of such follicles are much smaller in size than those of the old ones. In November the discharge and absorption of all unspent eggs are quite completed. In only exceptional cases can such cells be found at that time.

Orton (1933) found that in most cases the unspent eggs of *O. edulis* are extruded from the gonad within a few days after the principal spawning, while a small percentage of individuals may retain the remaining ova for as long as two months and then discharge them in the usual way. In the case of *V. mercenaria* the fate of some unspawned ova is probably similar to the fate of those in *O. edulis*. Studies of the gonads of *V. mercenaria* in post-spawning stages reveal that the number of undischarged eggs in the ovaries gradually diminishes as the season progresses. Some of the unspent ova found in the ovaries of clams during September–November are normal in appearance, having no indication of any decomposing processes going on. The fact that few if any phagocytes are observed in lumens of the ovarian follicles during the post-spawning period also adds weight to the conclusion that some of the ova unspent during the spawning season are extruded later in the normal manner. Such extrusion is completed in November.

Regardless of the fact that the temperature of the water over the clam bed decreases very rapidly during November, and in December falls below the point at which hibernation of clams begins, the growth and development of gametes proceeds normally. Ovaries of clams collected late in December already contain large numbers of ova which have a virtually mature appearance (Fig. 5). During that part of the season ovarian follicles contain relatively but very few of the minute ovocytes which were so characteristic for the gonads of early fall. It is quite clear that the young ovocytes which appeared in the follicles after completion of spawning have grown by now to a large size, and that since the appearance of the first and numerous group of ovocytes in September and October but few new cells appeared later on.

As may be seen from Fig. 5, in December samples large ovocytes protrude into the lumen occupying much of the available space in the follicles. A few pycnotic cells, some of them ovocytes in very early stages of growth, may often be noticed. It is very strange that during this time as well as the entire post-spawning period, when the ovogenetic activities of clams are at their height, very few nutritive cells are present near the follicles containing growing ovocytes. In the development of male gametes the rôle of such cells is of considerable importance since it is apparent that they participate in providing nutrition for the developing sex cells. In female clams ovogonia and ovocytes continue their growth mainly by extracting nourishment from the mesenchyme and blood vessels between the follicles. The nutritive cells, so important in male gonads, are much less numerous and apparently of lesser significance in the metabolic cycle of the ovary.



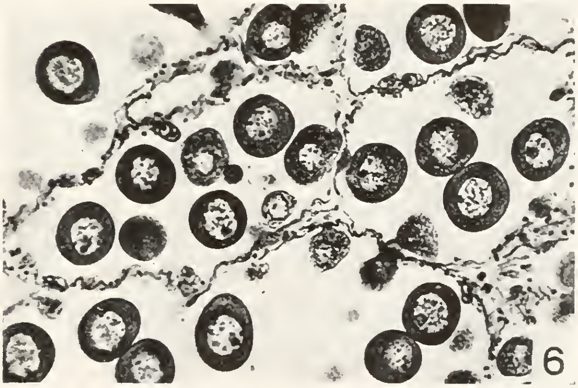


FIG. 5. Female gonad in December, containing large number of ova of mature appearance. × 300.

FIG. 6. Female gonad in May, containing ripe ova. × 300.

During the winter months, January–March, few changes occur in clam ovaries. The slow growth of young ovocytes continues but at a much reduced rate. There are few if any new young ovocytes beginning to develop from ovogonia during this time of the year. Large ovocytes, constituting the majority of the cells of the ovaries, measure up to 66–68 microns, i.e., equaling the size of the mature cells. Occasionally pycnotic cells may be noticed in one of the follicles, but as a rule there is little evidence of pycnosis. During April and early May the growth of the undersized ovocytes continues. By the middle of May the ovaries acquire a fully ripe appearance with approximately 90 per cent of all the cells reaching their limit in size which varies between 66 and 70 microns if the ovocyte is of normal oval shape.

During the second part of May some of the eggs of the clam are mature not only morphologically (Fig. 6) but also physiologically. On May 25, 1934, four large female clams were taken from the bed and brought to the laboratory where they were placed in an aquarium. The temperature of the water over the beds was 14.0° C. and that of the aquarium water 26.0° C. After two hours of exposure to such a temperature two females began spawning. Discharged eggs were fertilized by the addition of sperm suspension and they began to develop in the normal way. Thus, during the end of May, June and July, sexually mature and ripe clams merely wait for the water temperature to reach the point at which the proper stimuli inducing spawning reaction are produced. The general spawning of the clam population of the Charles Island beds occurs most frequently either late in July or early in August, and continues into September.

DISCUSSION

Studies of the changes occurring in the spermaries of adult *V. mercenaria* reveal that the post-spawning period, when the gonads are devoid of all but undifferentiated cells, is of very brief duration. This is probably the only period when the change of sex from male to female can take place. Production of a new crop of spermatozoa begins very soon after the completion of spawning. The main portion of spermatozoa for the next year's crop is produced during the autumn. Mature spermatozoa in small numbers may be found in the spermaries of a few clams as early as the end of September. In November the spermatozoa are already the most numerous cells in the gonad follicles, which at this time acquire an appearance of ripeness. Spermatogenesis continues at a reduced rate throughout the winter but is practically at a standstill during the early spring. The second manifestation of very rapid spermatogenetic activities takes place in June, when the water temperature reaches 15.0° C. The discharge of spermatozoa follows when the critical spawning temperature of 23–25° C. is reached.

Summarizing the observations of the processes occurring in the ovaries of *V. mercenaria,* it may be stated that the production of a new crop of eggs begins immediately after spawning. The chief growing period of the young ovocytes is in the autumn. In December and January the gonads of many female clams already present a ripe appearance. During the winter and spring only a few new ovocytes begin their development; cells produced in the autumn constitute the greatest portion of the next year's crop. Some ova, undischarged during the spawning season proper, may remain in the ovaries for a considerable length of time, and are finally discharged in the normal way. Many of the ova ripen approximately two and a half months before the water of the clam beds reaches the critical spawning temperature.

Compared with the sexual cycles of other pelecypods, it is rather unusual that in *V. mercenaria* the development of gametes occurs during the season when the water temperature rapidly decreases and the ecological conditions for gonad development appear to be less favorable than in the spring and early summer. According to Coe (1932), low temperature during the winter may delay the completion of the sexual phase in *O. lurida* for several months. A phase may be inaugurated in October and completed in April, while in the summer the same phase might be completed in a few weeks. The same author states that in the autumn ovulation in *O. lurida* is often inhibited. Judging by this it is probable that after the completion of the last phase of the year the animal does not display very active gametogenesis in the following autumn and winter, and that the main production of the gametes of the next sexual phase takes place in the spring when the water temperature begins to increase. In *O. gigas* (Amemiya, 1928) the production of gametes occurs chiefly in the spring. According to observations of the writer, adult individuals of *O. virginica* of Long Island Sound enter into the resting stage soon after completion of spawning and remain sexually inactive until the next spring. In *O. commercialis* (Roughley, 1933) the gonads are found in the resting stage throughout the winter and gametogenesis commences in the spring. Probably the same condition will be found in many other mollusks. In *V. mercenaria,* on the other hand, the most active gametogenesis and the production of the year's crop of sex cells occurs in the autumn and early winter. Since ovogenesis continues throughout fall and winter there is no actual period of recuperation which is commonly observed in other allied forms living in approximately the same geographical areas and subjected to similar ecological conditions. Retention of morphologically ripe sperm and ova throughout the greatest part of the year is another interesting feature of the sexual behavior of *V. mercenaria.*

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THE RATE OF WATER PROPULSION BY THE CALIFORNIA MUSSEL

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Numerous investigators have approached by means of various experimental techniques the problem of the volume of water which may pass in a given time through the mantle cavity of lamellibranch mollusks. The establishment of an approximate, or even a minimum figure for the volume of water pumped per day by any of the many marine plankton-feeding animals would be of interest to physiologists who may be concerned with nutritional, respiratory, or excretory activities of the animal itself, to planktologists whose problems deal with the numerous factors which influence the numbers and distribution of microscopic plants and animals that may be consumed by the filtering animals, and to oceanographers who seek information regarding biological factors concerned in modifications of the physical and chemical character of both water and bottom in various regions of the sea. In many instances, those who have contributed to our knowledge of the water-filtering and feeding activities of lamellibranchs have been primarily interested in the sanitary and other technical aspects of cultivating oysters and mussels for human consumption. Thus Viallanes (1892) presented approximate figures for the relative rates of filtration of water by French oysters, Portuguese oysters, and mussels. He placed the animals in separate crystallizing dishes upon the bottom of a tank supplied with flowing sea water, keeping initially empty dishes of the same size and design alongside of the experimental ones, in the same tank. At the end of a specified time interval the material which had been filtered from the water by the animals, and either swallowed and later voided as fecal material, or else rejected as pseudofeces directly from gill and mantle surfaces, was collected, dried, and weighed. The weight of filtered material was obtained by subtracting that of the detritus which had settled out of the water by gravity into the control dishes. Viallanes performed similar experiments more critically by dispersing a known quantity of dry clay in a given volume of water, drying and weighing the quantities of clay deposited by the different bivalves after 24 hours. From his first experiment, wherein the suspended material naturally present in the sea water was the only available particulate matter, he calculated that his animals (of age = 18 months) filtered water at the following relative rates: French oyster, 1.0; Portuguese oyster, 5.5; mussel, 3.0. The clay experiments, however, showed very different results, which were left undiscussed. Table I shows briefly his results.

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Viallanes found that the mucus which was secreted by the mollusks and in which the precipitated clay was incorporated, weighed only 4 per cent of the weight of the whole mass.

Ranson (1926) cites the work of Viallanes and reports briefly his studies of the mechanism of filtration. Both investigators emphasize the importance of the filtering function of lamellibranch plankton- and detritus-feeders to ostreiculturists and to oceanographic science.

Table I

Filtration and deposition of suspended clay by Viallanes' lamellibranchs.

Animal	Initial quantity of dry clay	Weight of clay deposited in 24 hours	Minimum volume of water filtered per hr. (our calculation)
	gram/liter	*grams*	*liters*
Mussel (18 months)	0.0546	1.768	1.35
Portuguese oyster (18 months)	0.0546	1.075	0.82
French oyster (18 months)	0.0546	0.199	0.015

Criticisms of Viallanes' experiments which must be borne in mind are: (1) in his first experiments with naturally-occurring particulate matter, the portion of the material which had been actually swallowed, the undigested residue of which was finally voided as fecal material, must have undergone changes of various kinds and degrees depending upon the relative rates and processes of digestion in the different species, and have subsequently vitiated the interpretations that were based merely upon relative weights; (2) in both of the experiments, the quantities of detritus or clay which had been removed from the water but still remained in the animals' alimentary tracts were not weighed or considered. Since in each case such small total weights were being measured, this item might have constituted a considerable, though by no means predictable or constant fraction of the total figure.

Galtsoff (1926, 1928*a*, 1928*b*) measured the rate of flow of water through the gill chamber of the American oyster, and calculated the work done by the propelling cilia. He used two methods, one a direct one in which the water issuing from the exhalant chamber was collected in a measuring vessel, the other in which the rate of advancement of a stream of water flowing from the exhalant chamber through a glass tube was measured by stop-watch. Both methods involved the insertion of a small glass rod between the opened valves to prevent their closing, and placing into the gill cavity a rubber tube, to carry off the discharged water, packing other open spaces around the tube with cotton. Galtsoff (1928*b*) records a maximum figure of about 3.9 liters per hour for the water intake of a single healthy adult oyster three to four inches in length at a temperature of 25° C.; the temperature at which the highest rate of flow occurred was found to be between 25° and 30° C. He points out, and shows by collected data the fact that considerable variations exist in the rate of flow produced by individual oysters. His average temperature-flow rate curve, taken from data collected in the study of many individuals, shows, at temperatures close to 30° C., a maximal rate of about 2.4 liters per hour while at 20° C. the average rate lies close to 2 liters per hour. At temperatures between 24 and 27.9° C., filtration took place at the rate of from 2.5 to 2.9 liters per hour. He points out that the filtering action of the oyster is dependent upon two mutually independent functions, namely the beating of the cilia and the opening and closing of the valves of the shell, the process occurring only when the valves are apart and the cilia beating. His studies showed that the oyster keeps its shell open for an average time of about seventeen hours out of twenty-four. (June to October.)

Numerous attempts have been made to estimate the rate of flow of water through the gill chambers of lamellibranchs on the basis of plankton counts in the water itself, and in the animals' stomachs. The results are, however, not satisfactory because, as Galtsoff (1928*b*) points out, there are daily and seasonal variations in plankton numbers in the water; not all of the diatoms filtered out by the gills are ingested but may be rejected instead. Such experimental work should, however, if carefully controlled, afford minimum figures. It would seem for various reasons probable that most methods used to date might be expected to yield results that are lower than the true figure for the volume of water filtered per day.

Various investigators (see also Galtsoff, 1928*b*) have obtained widely different results for the rate of water-pumping by plankton feeders. Collateral data regarding temperature are, however, not always available. Briefly, the general conclusions may be recorded as shown in Table II.

The inconsistencies which appear from an examination of these figures serve to emphasize the wide variations according to the method used, and the unreliability of the plankton count method. Galtsoff's studies convinced him that as many as 18.9 per cent of phytoplankton such as the diatoms *Chætoceros* and *Rhizosolenia*, and the dinoflagellates *Peridinium oceanicum* and *Ceratium* might escape being caught while passing through the gills, and that from 50 to 89 per cent of bacteria present in the water passed the gills of his oysters. Work at this Institution, however, shows that the mussel is quite successful in removing bacteria from water. Thus ZoBell and Landon (1937) demonstrated that the mussel removed about 99.9 per cent of added bacteria from sea water. (See also Fox et al., 1936.)

Without doubt, Galtsoff's direct methods are the most accurate for the measurement of the rate of flow of water through the mantle cavity under the conditions imposed upon the oyster. Needless to say, a question arises in one's mind regarding the possible influences that blocking the valves apart with a glass rod, inserting a rubber tube into the excurrent chamber, and packing the openings in the mantle surrounding the inserted tube with wads of cotton might exert upon the normal feeding and filtering behavior of the animal.

TABLE II

Filtration of water by plankton-feeders.

Investigator	Animal	Method	Average rate of filtration (liters per hour per animal)
Grave (1905)...	Oyster	Plankton counts in water and stomachs	0.167
Moore (1913)..	Oyster	Plankton counts in water and stomachs	ca. 1.25
Allen (1914)...	Freshwater mussel	Rubber tube packed into exhalant chamber	1.4
Wells (1916)...	Oyster	Plankton counts in water and stomachs	7.5
Nelson (1921)..	Oyster	Rubber apron dividing inhalant and exhalant chambers	5.7 (but see text below)
Galtsoff (1926, 1928*a*, *b*)....	Oyster	Rubber tube packed into exhalant chamber	2.5–2.9 at 24–26.9° C.
Dodgson (1928)	Mussel (*Mytilus edulis*)	Clearing of suspensions	2.0 (minimum, at 17° C.)
Parker (1914)..	Sponge (*Spinosella*)	Glass tube tied into osculum	3.2
Damas (1935)..	Cardium	Clearing of mud suspensions	0.1

Nelson (1935) refers to earlier efforts to measure the water filtered by an oyster, and to the fact that very diverse results were obtained. He writes, " The introduction of a tube into the cloacal chamber interferes with normal operation of the branchial hearts described by Hopkins and may disturb the visceral ganglion." He adds, " Also, in *Ostrea virginica* much of the water from the right demibranch leaves by an asymmetric chamber on the right side and separate from the cloacal chamber." Using a modification of the rubber apron of Moore (1908), he claims to have measured all the water passed by an oyster, without interfering with its normal activities. He reports the amazing value of 26 liters of water per hour, passed by an oyster 11.5 × 8.9 cm. in size, at the optimum temperature of 30° C. This is about tenfold the average values found by Galtsoff for the oyster and by ourselves for the mussel. Fresh oyster sperm were found by Nelson to increase markedly the rate of water propulsion by male oysters, while in females no response was observed unless spawning occurred, in which case the filtration-rate was temporarily reduced.

Parker (1914) reports on the strength and volume of water currents produced by sponges. He measured the average height to which the excurrent stream of water might reach when glass tubes of appropriate size were tied securely into an osculum. By measuring the rate at which carmine particles, etc., were carried out of a glass tube of known dimensions, Parker concluded that the sponge *Spinosella* discharged water from its oscula at a rate of about 4.5 cc. in five seconds, or about 78 liters per day. A colony of *Spinosella* having as many as twenty oscula might, he concluded, strain in a day more than 415 gallons of water.

Damas (1935) studied the activities of plankton-feeders, especially *Cardium* and other lamellibranch mollusks, with reference to their rôle in the deposition of marine muds. He calculated, on the basis of quantitative observations of the extraordinary rate of deposition of mud pellets by *Cardium*, that 1,000 such individuals produce, on an average, a layer of mud of 0.45 meter in thickness per square meter per year, or 1,250,000 cubic meters of mud per year in the 250 hectares (1 hectare = 10,000 sq. m.; 2.471 acres) colonized by *Cardium* in the roadstead of Zeebrugge.

The question of the natural filtration rate has been approached by Dodgson (1928) and by ourselves under conditions which would seem to simulate more closely those of nature. Dodgson and co-workers, working with the bay mussel, *Mytilus edulis,* at Conway, Wales, prepared turbid suspensions of different substances such as flour, clay, fine silt, and even ordinary muddy river water, in sea water. On the basis of many experiments, Dodgson claims that " end-points," i.e. the time at which the formerly cloudy solutions become quite limpid, could be determined without difficulty, the last traces of turbidity disappearing almost suddenly. At 17° C., mussels placed in turbid suspensions of fine silt, mud, clay, or flour filtered the water to clarity at estimated minimum rates varying between 1.9 and 2.6 liters per hour per mussel. His average figure of about 2 liters per hour per mussel is expressed as being probably far less than the actual quantity, since, in order to remove quantitatively a suspended substance from a given mass of water, the animal must, because of the constant mixing of the filtered with the unfiltered water, pass some of the water through its mantle cavity many times over, even should the water issuing from the exhalant siphon be completely cleared.

EXPERIMENTAL

In our work with the California sea mussel, *Mytilus californianus,* which unlike the *M. edulis* that inhabits bays, estuaries and river mouths, attaches itself to rocks and pilings near open, unprotected shores, we used some refinements of Dodgson's general method. We considered that serial measurements of a finely divided substance remaining suspended in a given mass of water in which mussels were immersed should provide approximate data regarding the rate of filtration of the water by the animals.

We have not overlooked the fact that individual animals may vary considerably in their relative rates of propelling water through their gill chambers; we have also considered the possibility that the rate of filtering by the animal may depend to a considerable extent upon such factors as (*a*) particle size, (*b*) concentration and (*c*) chemical nature of the chosen suspended material (i.e. whether of nutritional, inert, or injurious character), and the possible influence of such properties upon mucus secretion, ciliary motion, and frequency of closure of the valves.

For convenience in analyzing at intervals remaining suspended material, and in order to duplicate to some extent the conditions of nature, use was made of a calcareous marine mud, whitish to light grey in color, from Bird Key Harbor, Florida. In preliminary experiments [2] use was made of material which, according to Dr. E. M. Thorp, had passed through a 0.48-mm. mesh screen. Its introduction into water in which mussels were immersed had no perceptible influence upon the animals, which remained with valves apart and continued to filter water. Mussels when kept out of water for a short interval, then placed in a very turbid suspension of the mud, opened their valves without delay, and began "pumping" water. Figures 1–4 show a series of photographs illustrative of the rather striking clarification of turbid water in a relatively short time. Into each of two graduated cylinders, each containing

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[2] These were undertaken in the early summer of 1935 by the senior author in collaboration with Dr. Roderick Craig of the Division of Entomology and Parasitology, University of California.

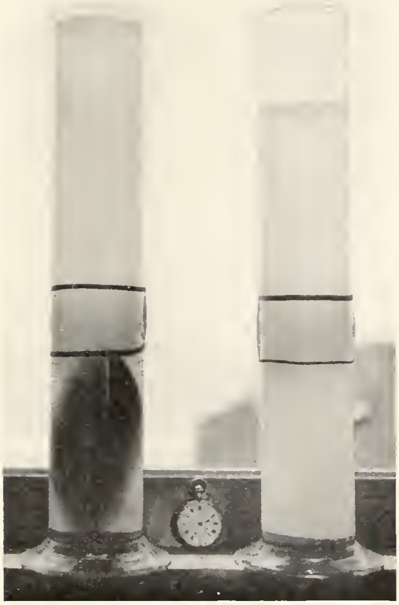

FIG. 1.

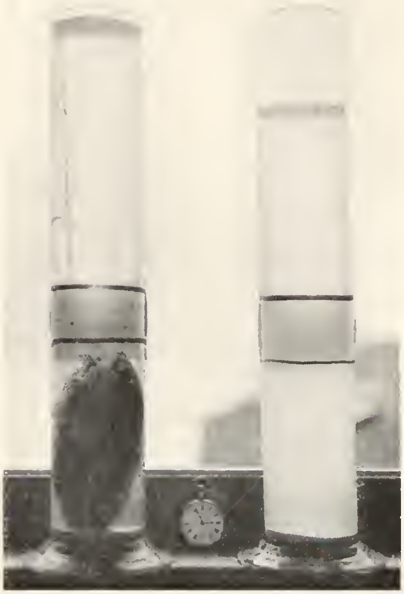

FIG. 2.

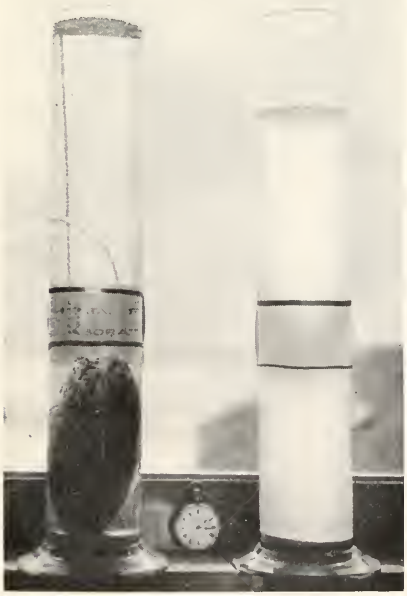

FIG. 3.

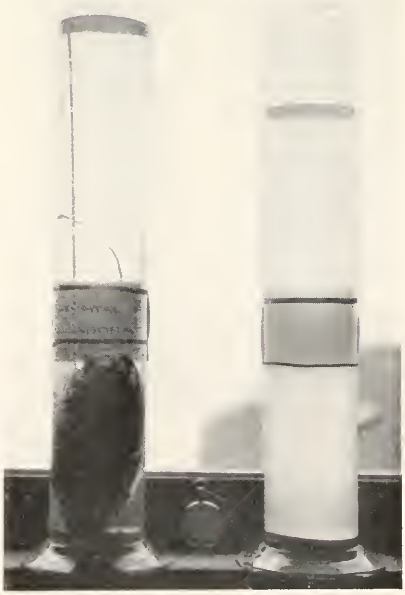

FIG. 4.

FIGS. 1–4. Removal of mud from suspension by a mussel. (Photographs by Dr. R. Craig.)

2,000 cc. of sea water, were placed 20 grams of the calcareous mud which was mixed with the water by several inversions of the temporarily stoppered containers. After the coarsest particles had settled out within a few minutes, the containers were placed in a window to afford good lighting, and a large mussel of 17 cm. length, and weighing about 485 grams, was placed in the one on the left side. The first photograph shows an identical degree of turbidity in the two suspensions at the beginning, as noted in the faint Tyndall beam from the edges of the cards strapped at the rear outer wall of each cylinder. In half an hour (Fig. 2), the turbidity in the mussel-containing jar had been greatly decreased, and the picture shows the printed letters upon the card, easily legible through the water. In 50 minutes (Fig. 3) the turbidity in the left container was all but gone, and in the last of the series (1 hour and 45 minutes) we see a perfectly clear solution. Note particularly the serial appearances of the string by means of which the animal had been lowered slowly into the container. The temperature of the collected sea water was initially about 20° to 21° C., and could have increased by not more than a degree or two.

Inspection of the sediment at the bottom of containers on the day following a filtration experiment revealed three types of precipitated mud: (1) very fine, homogeneous powder which had settled out by gravity; (2) piles of pseudofeces in the form of amorphous, stringy masses of material which had been filtered out by the mussel, incorporated in mucus, and expectorated from the mantle at the excurrent siphon; the extrusion of this material could be observed continuously from the beginning of the experiment; (3) true characteristic feces in the form of short, discrete, flattish straps composed of the mud which had been ingested by the mussel.

The intricate arrangements possessed by lamellibranchs for filtering the fine detritus and small organisms from water have been described and discussed extensively by other authors (Moore, 1905; Allen, 1914; Kellogg, 1915; Dodgson, 1928; Yonge, 1928; and others cited especially by the latter).

We know from the experiments reported in this paper and from previous ones (Fox et al., 1936) that the material filtered by the mussel from water may be in part swallowed (even if inert and nutritionally useless) and in part expectorated in mucus-laden strings or pseudofeces from the edges of the mantle, especially if the water contains a great amount of suspended matter. We were not in this particular study interested primarily in the relative quantities that were swallowed or rejected; we were interested in the rate of diminution of suspended material, and made preliminary measurements [3] in order to learn (1) how rapidly water may be propelled through the gill chamber of the mussel during such metabolic activities as feeding, respiration and excretion, and (2) how rapidly suspended organisms or other material may be removed from water, to be ultimately deposited in an altered state upon the bottom of the sea.

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[3] In the late summer of 1935 by the senior author jointly with Mr. Rae Schwenck, from the Department of Chemistry, Sacramento Junior College.

The results of these preliminary experiments were, however, not entirely satisfactory. Their difficulty of interpretation was doubtless due to the fact that we were at that time unaware of all the precautions which had to be taken in order to obtain results which could be readily analyzed.

The experiments were therefore repeated in the fall of 1936 (D. L. F. and J. P. C.), with the introduction of several refinements in technique and materials; these experiments will here be dealt with more fully. In the first of them the same calcareous mud was employed, but of a much finer grade, having passed (according to Dr. Thorp) through a 0.086 mm. mesh screen. The dry mud was shown by analysis to contain 1.66 per cent moisture, 90 per cent $CaCO_3$, and 6 per cent SiO_2 (by difference). In later experiments, we used pure $CaCO_3$ instead, and found that it served equally well. (See below.)

In our experiments 8,000 cc. of fresh sea water, to which were added initially 32 grams of mud (4 grams per liter of water), were placed in each of a series of large battery jars. The suspensions were allowed to stand for about one hour, to allow time for larger aggregates of mud particles to settle to the bottom.

The mussels were handled carefully throughout the work; they were placed on the table for a time with the valve openings vertical to the surface, in order to allow the water within the mantle cavity to drain out when the animals opened their shells; this not only prevented changes in the volume of water introduced into the containers, but probably rendered the animals sufficiently " thirsty " to insure their commencing activities almost directly they were immersed in the suspensions; the prompt opening of their valves after immersion could be recognized by bubbles of displaced air rising to the surface, and often also by watching the animals which lay close to the glass walls of the jars. A moderate stream of air was introduced through a glass tube reaching to the bottom of each jar, sufficient to insure constant homogenous mixing without disturbing the animals or stirring the heavier material which was deposited on the bottom by ordinary settling or by the animals.

Although the animals kept their valves apart during the course of the experiments, it was observed, by following the course of suspended particles, that the currents of water flowing into the incurrent and out of the excurrent siphon were not of continuous intensity, but occurred at apparently rhythmical intervals, the intensity of the stream alternately increasing to a maximum, then gradually diminishing to a minimum. This could be observed particularly well in the large mussels. Mucus-laden strands of the filtered mud were expelled at a slow but

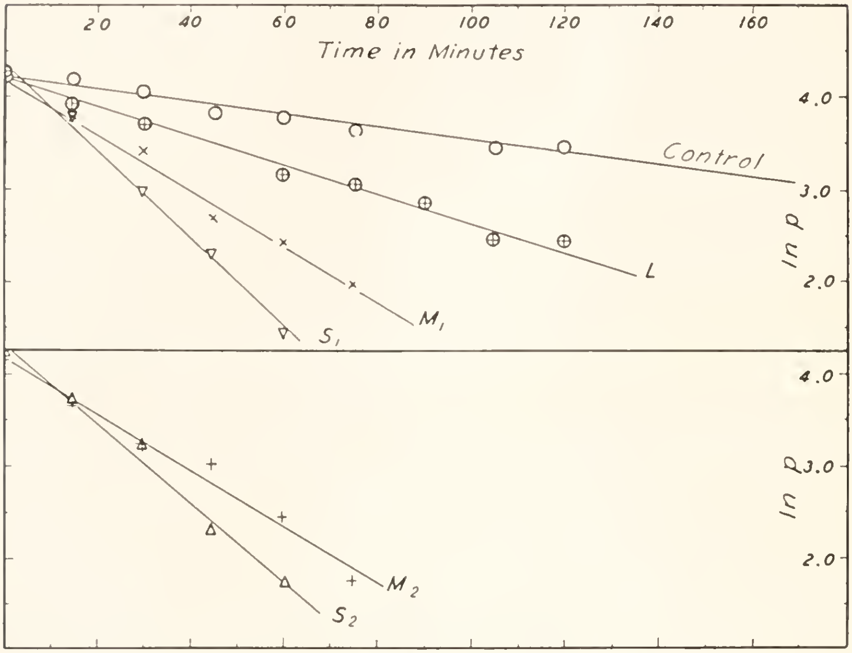

Fig. 5. Removal of suspended $CaCO_3$ by mussels; first series.

First Series:

Large mussels—	2 animals. Lengths 130 mm. and 110 mm. (average = 120 mm.). Total weight 333 grams (average = 166.5 grams) each.
Medium mussels—(M_1)	4 animals. Lengths 97, 100, 100 and 103 mm. (average = 100 mm.). Total weight 302.5 grams (average = 75.6 grams) each.
(M_2)	4 animals. Lengths 95, 95, 105 and 105 mm. (average = 100 mm.). Total weight 299.4 grams (average = 74.8 grams) each.
Small mussels—(S_1)	9 animals. Lengths 70, 78, 67, 73, 76, 72, 75, 74 and 65 mm. (average = 68.9 mm.). Total weight 308.4 grams (average = 34.4 grams) each.
(S_2)	9 animals. Lengths 78, 69, 79, 75, 82, 79, 60, 62 and 82 mm. (average = 74 mm.). Total weight 360.0 grams (average = 40 grams) each.

Ten-cc. samples taken every 15 minutes. Temperature range 22.85° to 23.4° C.

nearly constant rate from the edges of the mantle at the exhalant opening. These pseudofeces either fell rapidly to the bottom, or, if attached to an air bubble or two, rose to the top. They did not break down and become redispersed.

Samples were removed at stated intervals (10 or 15 minutes) from a uniform place in the center of the container and at about the mid-depth point, with calibrated 10-ml. pipettes, and introduced into stoppered vessels for analysis. These samples were acidified before washing them into containers for analysis, in order to insure that none of the colloidal $CaCO_3$ material remained adsorbed to the walls of the vessels. The microchemical method of Kirk and Moberg (1933) for the analysis of calcium in blood or sea water was employed. The calcium is precipitated as the oxalate, washed, and titrated in acid solution with standard potassium permanganate. By this method the amount of calcium is determined with an accuracy of ± 4 mg./liter. The concentration of dissolved calcium in the ordinary sea water used in these experiments was determined by the same method.

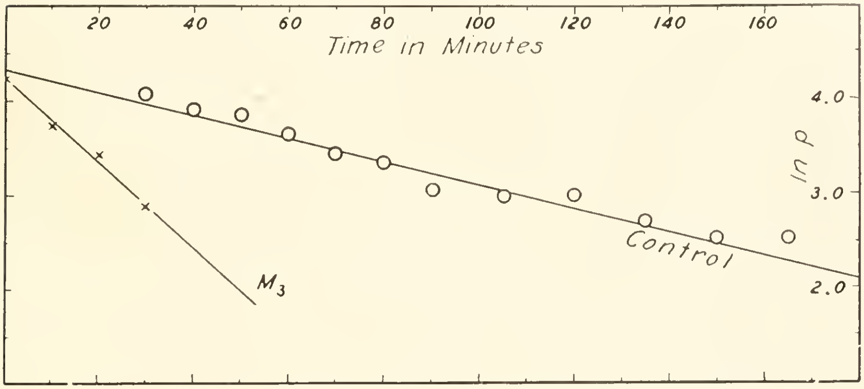

FIG. 6. Removal of suspended $CaCO_3$ by mussels; second series.

Second Series:

Six mussels used.
Medium (M). Lengths 98, 108, 100, 103, 105, 107 mm. (average = 103.5 mm.).
Total weight 611.5 grams (average = 102 grams).
Ten-cc. samples taken. Temperature range 20.8° to 21.35° C.

Controls (i.e. jars containing identical quantities of water and suspended mud, with similar moderate aeration but without mussels) were always carried out, parallel with the experiments, and samples were taken from these at the same intervals.

Two series of experiments (Numbers 1 and 2) were undertaken using the calcareous marine mud; the results are shown in Figs. 5 and 6.

The data for these figures were collected and originally tabulated in exactly the way shown in Table III.

Two series of experiments (Numbers 3 and 4) were also performed with the use of pure, finely divided $CaCO_3$; the results are recorded in Figs. 7 and 8 (from data tabulated as in Table III, which shows data from which Fig. 8 was derived). Freshly collected specimens were used in these experiments. Placed carefully in the containers, they soon opened their valves, and showed no objection whatever when the carbonate was added, but continued to filter the water in a normal manner.

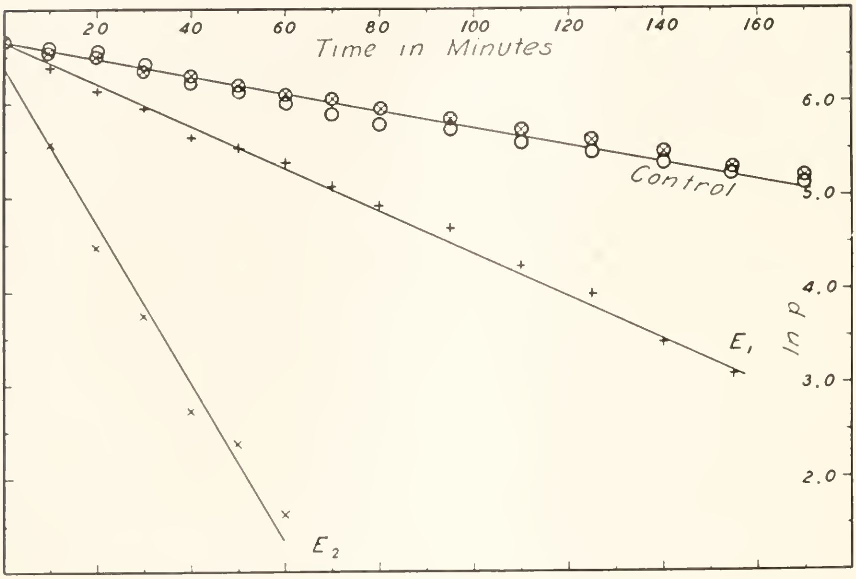

Fig. 7. Removal of suspended $CaCO_3$ by mussels; third series.

Third Series:

Very large mussels.

E_1. Two animals. Lengths 179 and 174 mm. (average = 176.5 mm.). Weights 434.7 and 395 grams respectively (average = 415 grams).

E_2. Two animals. Lengths 182 and 178 mm. (average = 180 mm.). Weights 337 and 400 grams respectively (average = 368 grams).

Ten-cc. samples taken. $CaCO_3$ used: 2 grams/liter (16 grams total) in each tank. Temperature range 18°–20° C.

In one experiment (see Fig. 7) two pairs of very large mussels, collected at an exceptionally low tide, were employed. These individuals were selected from the catch on the basis of fair uniformity of size and their readiness to open their valves and propel water without showing cessation of activity or other disturbed responses to slight mechanical stimuli such as stirring the water in which they were immersed, or tapping upon the container.

Four jars were set up, each containing 6 liters of sea water. Two selected mussels were placed in each of two jars while the other jars were left as controls. Air was passed at a uniform rate through each jar of water as before. After all four of the animals had parted their valves, the additional two liters of water were added to each jar (to make up the total of 8 liters), the suspended $CaCO_3$ being added with the water in the operation.

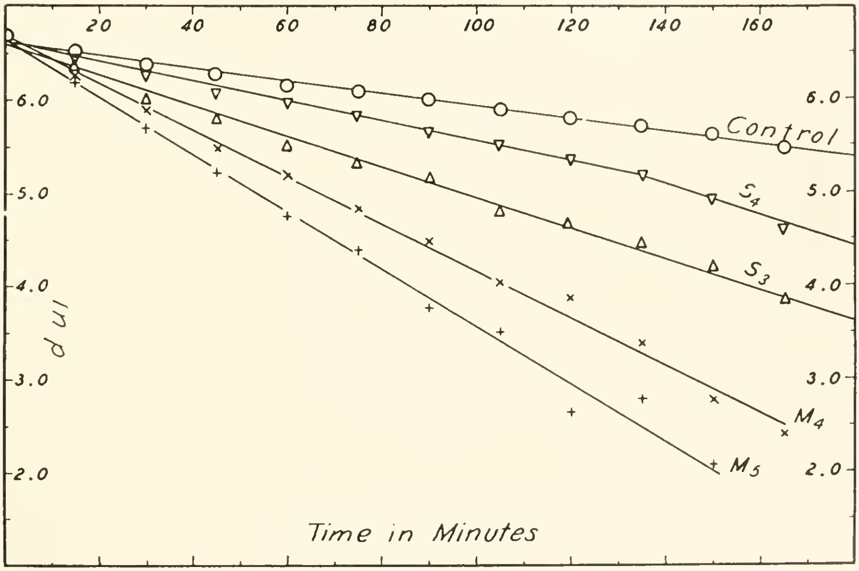

Fig. 8.

The "zero points" of the two control jars (without mussels), which agree very closely, were obtained by taking samples immediately after adding the $CaCO_3$ in the regular manner and are taken to serve as initial points for the duplicate experiments as well, since identical quantities of the salt had been added to the same volumes of water under the same conditions, and previous experiments had shown good checks in the initial quantities of suspended material under such conditions.

Table III shows data relating to duplicate experiments on (1) medium-sized mussels (M_4 and M_5) and (2) small mussels (S_3 and S_4) all run simultaneously along with a control (*C*). Four mussels were placed in each jar, and the experiments were conducted in a manner identical to the previous set. Again, the initial value for suspended $CaCO_3$ in the *control* jar was taken to represent a reliable figure for that of the experimental jars.

In these experiments wherein $CaCO_3$ was used, it is noted that although the initial quantity of $CaCO_3$ suspended in the water exceeds by an average of some ten-fold the quantity suspended when the calcareous marine mud was employed in the earlier experiments, nevertheless the rate of removal of suspended material by mussels of from 69 to 105 mm. in length is similar in both series of experiments. (See Table IV.)

TABLE III

Removal of suspended $CaCO_3$ by mussels—fourth series. Medium mussels: M_4—4 animals. Lengths 105, 100, 102 and 109 mm. (Average = 104 mm. each). Total weight 342.5 grams (Average = 85.6 grams each). M_5—4 animals. Lengths 106, 104, 102 and 108 mm. (Average = 105 mm. each). Total weight 337.5 grams (Average = 84.4 grams each). Small mussels: S_3—4 animals. Lengths 78, 79, 77 and 74 mm. (Average = 77 mm. each). Total weight 139 grams (Average = 34.75 grams). S_4—4 animals. Lengths 75, 81, 74 and 77 mm. (Average = 77 mm. each). Total weight 156 grams (Average = 39 grams each). Ten-cc. samples taken. Temperature range 19°–20.3° C. $CaCO_3$ used. Two grams per liter (Sixteen grams total in each tank).

Time	Minutes	Control C	Medium M_4	Medium M_5	Small S_3	Small S_4
		Ca	Ca	Ca	Ca	Ca
		grams/liter	*grams/liter*	*grams/liter*	*grams/liter*	*grams/liter*
1:15 P.M....	0	1.2356	—	—	—	—
2:10 P.M....	15	1.1185	.9542	.9314	1.0142	1.1000
2:25 P.M....	30	1.0171	.7828	.7271	.8385	.9542
2:40 P.M....	45	.9585	.6714	.6142	.7571	.8642
2:55 P.M....	60	.8928	.6057	.5457	.6771	.8214
3:10 P.M....	75	.8685	.5543	.5114	.6371	.7642
3:25 P.M....	90	.8242	.5185	.4728	.6000	.7200
3:40 P.M....	105	.7828	.4871	.4628	.5500	.6814
3:55 P.M....	120	.7528	.4771	.4443	.5343	.6428
4:10 P.M....	135	.7300	.4585	.4457	.5143	.6085
4:25 P.M....	150	.7028	.4457	.4385	.4957	.5671
4:40 P.M....	165	.6685	.4414	.4243	.4771	.5314
4:55 P.M....	180	.6500	.4343	.4257	.4685	.5143

The total calcium content represents the sum of the amount of calcium which is present in solution in the sea water and the added amount which is present in suspended material. The decrease of the calcium content with time is due to:

1. Gravitational settling of suspended material.
2. Removal (filtration) of suspended material by the mussels.

In the control experiments, which were undertaken without mussels in the vessel, the decrease was due to gravitational settling only, and a general law can be found for the rate of this settling. During the experiments with mussels in the vessel, the decrease was due partly to settling and partly to removal of the material from suspension by the mussels; knowing the former, the rate of removal of the calcium by the mussels can be determined by difference. In all cases the mussels decreased the calcium content to a minimum value of about 430 mg. Ca per liter, whereas a determination of the contents in water filtered through paper gave about 440 mg. Ca per liter. Additional unpublished experiments have shown that a small amount of the calcium in the water, present probably in a very finely suspended state, could be filtered by the mussels but was not filtered out by paper alone, previous to analysis. It appears therefore justifiable to assume that the content in solution was about 430 mg. Ca per liter and that *the suspended amount was equal to the observed value minus 430 mg. Ca per liter.* The value 430 may be in error by about 1 mg./liter whereby a slight uncertainty is introduced as to the amount of suspended calcium, and this uncertainty exerts a corresponding slight influence upon the further treatment of the data, since this has to be based upon the *suspended* amount of calcium and not upon the total amount.

TABLE IV

Liters of water passed per mussel per hour.

Series No. and value of a	Experiment No.	Size of mussel		Number of mussels	Value of b	m in liters per hour
		Mean weight	Mean length			
		grams	*mm.*			
(1) 0.39	1	166.5	120	2	0.93	2.2
	2	75.6	100	4	1.81	2.8
	3	74.8	100	4	1.82	2.9
	4	34.3	69	9	2.80	2.1
	5	40.0	74	9	2.57	1.9
(2) 0.70	6	101.9	103.5	6	2.59	2.5
(3) 0.54	7	415	176.5	2	5.05	18.1
	8	368	180	2	1.36	3.3
(4) 0.41	9	85.6	104	4	1.51	2.2
	10	84.4	105	4	1.85	2.9
	11	34.8	77	4	0.98	1.1
	12a	39.0	77	4	0.65	0.5
	12b	39.0	77	4	1.02	1.2

By plotting the results from the control experiment one obtains a curve of a form which suggests that in the absence of mussels the amount of suspended calcium can be expressed as an exponential function of time. We assume, therefore, that the amount which is precipitated in unit time is proportional to the total amount which is suspended. If we have in the vessel M liters of water containing p mg. of suspended calcium per liter, the amount precipitated in unit time will be aMp, where a is a factor of proportionality. The amount precipitated in the time dt will be:

$$(a\ M\ p\ dt)$$

Concerning the action of the mussel, we will assume that *in unit time each mussel pumps* m *liters of water through its system and removes all suspended calcium from this quantity of water.* The amount removed by one mussel in unit time will then be $m\ p$, and if we have n mussels the amount of suspended calcium removed in the time dt will be:

$$(n\ m\ p\ dt)$$

The total amount removed owing to both ordinary precipitation and to the action of the mussels, representing the total decrease of the suspended amount of calcium, is therefore:

$$d(M\ p) = -\ (nm + aM)\ p\ dt \tag{1}$$

where the minus sign indicates a decrease. Therefore;

$$\frac{dp}{p} = -\left(\frac{nm}{M} + a\right) dt = -\ bdt$$

$$\ln p = -\left(\frac{nm}{M} + a\right) t = -\ bt \tag{2}$$

$$p = p_0 e^{-\left(\frac{nm}{M}+a\right)t} = p_0 e^{-bt} \tag{3}$$

The logarithmic decrement a can be determined from the control experiment and the logarithmic decrement b from the experiments with mussels. The amount of water which passes through one mussel in unit time is then:

$$m = M\,\frac{b - a}{n} \tag{4}$$

If our assumptions are correct, each experiment must reveal a linear relationship between the natural logarithm of p (the amount of suspended calcium in mg./liter) and the time. Furthermore, we must obtain nearly the same values of p_0, the amount of suspended calcium at the beginning of the experiment, since in each case the initial conditions were as similar as possible.

In order to examine this question, the values of p have been computed by subtracting 430 mg./liter from the observed values of the total calcium content. All values of p smaller than 5 mg./liter have been omitted (except in one instance when a value of 4.3 mg./liter has been retained) since, owing to the slight uncertainty involved in the micro method for determining the amount in solution (430 mg./liter), small values of p have no significance. In the case of the control experiments values only from the first three hours have been considered, although p remained greater than 5 mg./liter.

In Figs. 5, 6, 7, and 8, the values of $ln\ p$, as derived from the four series of experiments, have been plotted against time. The straight lines represent lines of regression which have been computed by the method of least squares. It is seen that all points fall so near to the lines that the deviations may be considered accidental. Accidental deviations must arise, since the samples taken from the middle of the jar cannot be expected to show exactly the average contents of suspended calcium, and since errors of determination are present. In the case of the small mussels (S_4), in series No. 4, a break in the line appears to occur after 130 minutes, indicating that the rate of propulsion of water through the mussels suddenly changed.

From the control experiments, we obtain various values of our constant a, using one hour as the unit of time (see Table IV). The variation of the values of a may be due to differences in the velocity of the stream of air which was passed through the water in order to insure homogeneous mixing, or, to a lesser extent, to differences in the effect of the salt water in coagulating the suspended material. In all experiments, however, the velocity of the air stream was adjusted so that the rate of bubbling through control jars and experimental jars was as closely identical as estimation would permit.

From the experiments with mussels we find the values of b which are shown in Table IV and the values of m (in liters per hour) which have been computed by means of formula (4), introducing M (8 liters), the number of mussels (n), and the value of a which was found by simultaneous control experiment.

The fact that in all cases we find a linear relationship between $ln\ p$ and time appears to furnish strong evidence for the correctness of our assumptions, but from our equations it is evident that we should obtain a linear relationship on other assumptions than these which have been introduced. We could assume that the mussels remove only a constant fraction of the suspended calcium when m liters pass through the mussel. The amount removed would then be $cnm\ p$, where c must be supposed to be a constant factor smaller than 1.0. It appears, however, improbable that a mussel should remove, say, 50 mg./liter when the content was 100 mg. liter and in the same interval of time, 2 mg./liter when the content was 4 mg. liter. A change in the ratio of the removed calcium from say 80 per cent of a high content to 100 per cent of a small content is, however, possible and would not be detected. If such a change takes place, our values of m are conservative, being somewhat low. Another conceivable possibility which may be mentioned is that the amount of water passing through the mussel chamber might decrease with time and the fraction of calcium removed might increase (or vice versa). However, the product mp must, since $ln\ p$ is a linear fraction of time, remain nearly constant, and this means that the amount of water passing through the mussel chamber and the fraction of calcium removed would have to change in opposite directions at the same rate. It is difficult to conceive a mechanism which would work in such a manner. The simplest explanation of our results appears to be that our original assumptions are correct, viz., that a constant amount of water passes through the mussel chamber in unit time and that virtually all suspended (and colloidal) calcium in this amount of water is removed.

We have furthermore supposed that the water passing through the mussels has the average calcium content of the water in the vessel. In order to satisfy this condition, the water must be stirred, since the mussels lying on the bottom of the vessel remove practically all suspended calcium from the water passing through them. The stirring must be adapted to the number of mussels; the greater this number the more rapid must the stirring be. The effect of insufficient stirring upon the observations would be that $ln\ p$ would no longer be a linear function of time, but would decrease more and more slowly as the experiment advanced. The reason for this is that the amount of suspended calcium removed by the mussels would no longer be proportional to p, but since the bottom layer, where the mussels lie, would be depleted of calcium more rapidly, it would be proportional to some function of p which decreases with time. We can introduce

$$nm\ p\ e^{-ct}\ dt$$

instead of

$$nm\ p\ dt$$

and obtain

$$\ln p = \ln p_0 - at - \frac{nm}{Mc}(1 - e^{-ct})$$

At great values of t, we obtain

$$\ln p = \ln p_0 - \frac{nm}{Mc} - at,$$

meaning that the decrease in the calcium content approaches the value which is due to gravitational settling only. The coefficient c which has been introduced depends upon the rate with which water passes through the mussels and upon the rate of stirring, and a determination of this coefficient is hardly possible. A computation therefore cannot be based upon the results of such experiments. During the experiments which are dealt with here, the water was stirred by passing through a constant stream of air, and the agreement between observed and computed values shows that the stirring was sufficient.

Two preliminary experiments which Fox and Schwenck undertook in the summer of 1935 with two very large mussels weighing 485 and 515 grams show a wider scattering of the observed values, but they indicate a linear relationship between *ln p* and *t*, giving *m* equal to 1.8 and 2.4 liters per hour respectively.

TABLE V

Rates of water propulsion by mussels.

Weight of mussels		Length of mussels		Number experiments	Total no. mussels used	Rate of water propulsion	
Range	Average	Range	Average			Range	Average
grams	*grams*	*mm.*	*mm.*			*liters/hr.*	*liters/hr.*
337–515	431	174–182	178	4	6	1.8–18.1	6.4
75–166	93	95–130	102	6	24	2.2– 2.9	2.6
34– 40	37	60– 82	74	4	26	0.5– 2.1	1.4

Other preliminary experiments by Fox and Schwenck, in the summer of 1935, gave less consistent values. In these experiments a large number of small mussels were used and *ln p* decreased rapidly at the beginning, but slowly at the end of the experiments, probably because the stirring was insufficient.

The results of all experiments, including the preliminary ones by Fox and Schwenck, are summarized in Table V. The very large mussels show an enormous range in their rate of water propulsion, perhaps owing to individual differences or perhaps because they work intermittently. The value obtained from one of the experiments, 18.1 liters per hour, is quite enormous, but it is undoubtedly correct since it is based on good observations during one full hour, and it shows that in certain circumstances large mussels can pump great quantities of water through their systems. The average value for the large mussels, 6.4 liters per hour, cannot be given any weight owing to the wide range of the single values and the small number of experiments. The medium-sized mussels appear to be more consistent in their behavior, the range of the single values is small and the average value, 2.6 liters per hour, can therefore be considered nearly correct. The small mussels show again a wider range of their rate of water propulsion and while the average value is uncertain, it undoubtedly lies below that of the larger animals.

It would be expected that both the weight and the volume (capacity of the gill chamber) of the mussel should be proportional to some fairly constant power of one of the linear dimensions, say the length of the shell, and that weight should show a linear relationship to capacity. The capacity (in ml.) was determined by allowing mussels whose gill chambers had been emptied of sea water to refill their cavities on immersion in a known volume of sea water, then causing the animals to close their valves, removing them from the vessel, and measuring the residual water in the container. Plotting the average lengths of a series of different sized animals (in mm.) against their capacities (in ml.) showed a steeply rising curve of an exponential character; length plotted similarly against weight of animals (in grams) emptied of water showed a curve of similar character (see also Galtsoff, 1931, who obtained a similar relationship in the Hawaiian pearl oyster); finally the relationship between weight and capacity was shown to be a linear one.

An extensive series of experiments performed upon mussels of different known capacities, weights, and linear dimensions would perhaps disclose an interesting relationship between any of these attributes (say the volume), and the rate of propulsion of water by each size of " pumping system."

SUMMARY

A method is described for determining the approximate average rate at which the California mussel, *Mytilus californianus,* propels water through its gill chambers.

The method consists of analyses at frequent intervals of the amount of calcium remaining in suspension (as $CaCO_3$) in a given volume of continually stirred water containing the mussels, which remove the suspended material as they pass the water through their chambers.

Mathematical treatment and interpretation of the data obtained, support the conclusions that (1) virtually all of the suspended matter is removed as the water passes over the mucous surfaces of the gills and mantle of the mussel; (2) the mussel propels the water rhythmically

through its filtering system at a rate which on an average is constant, varying according to the size and perhaps also according to other physiological attributes of the animals. In medium-sized animals (of 95 to 130 mm. length) the propulsion rate may vary between extreme values of 2.2 and 2.9 liters per hour, and has an average value of approximately 2.6 liters per hour.

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INDEX

ABRAMOWITZ, ALEXANDER A. The chromatophorotropic hormone of Crustacea, 344.
Acanthephyridæ, eyes of, 57.
Accelerator substances, evidence for production by ultraviolet radiation of Limulus muscle, 75.
Allee, W. C., and Gertrude Evans. Some effects of numbers present on the rate of cleavage and early development in Arbacia, 217.
Amœba proteus, mitosis in, 125.
Amphiscolops langerhansi, reproductive system and copulation, 319.
Arbacia, cleavage rate, as affected by numbers present, 217.
Arbacia punctulata, prolongation of life in egg cells and calcium reduction, 366.
Arey, Leslie B. Observations on two types of respiration in Onchidium, 41.
Aurelia, budding and locomotion in scyphistomas of, 99.

BACTERIA, and phosphorus cycle in the sea, 190.
Beadle, G. W. *See* Clancy and Beadle, 47.
von Brand, Theodor. Observations upon the nitrogen of the particulate matter in the sea, 1.
— ——, ——, Norris W. Rakestraw and Charles E. Renn. The experimental decomposition and regeneration of nitrogenous organic matter in sea water, 165.
Budding and locomotion in scyphistomas of Aurelia, 99.

CALANUS finmarchicus, feeding rate of, in relation to environmental conditions, 233.
Calcium reduction and prolongation of life in egg cells of Arbacia punctulata, 366.
Cameron, John Andrew. The mitotic rate in tadpole skin after repeated injury, 37.
Caterpillars, gustation and olfaction in, 7.
Centrifuging, behavior of maturation spindles in polar fragments of Ilyanassa eggs after, 88.
Chace, F. A., Jr. *See* Welsh and Chace, 57.
Chase, Hyman Y. The effect of ultraviolet light upon early development in eggs of Urechis caupo, 377.
Chromatophorotropic hormone of Crustacea, 344.
Churney, Leon, and Herbert M. Klein. The electrical charge on nuclear constituents (salivary gland cells of Sciara coprophila), 384.
Clam, adult, seasonal gonadal changes, 406.
——, development of primary gonad in, 389.
Clancy, C. W., and G. W. Beadle. Ovary transplants in Drosophila melanogaster: studies of the characters singed, fused, and female-sterile, 47.
Cleavage, rate in Arbacia, effects of numbers present, 217.
Color-changes, and chromatophorotropic hormone of Crustacea, 344.
—— ——, and diurnal rhythm in Ligia baudiniana, 24.
Coonfield, B. R. Symmetry and regulation in Mnemiopsis leidyi, Agassiz, 299.
Cunningham, John P. *See* Fox, Sverdrup and Cunningham, 417.
Cyst formation in glomerular tufts of certain fish kidneys, 247.

DALCQ, A., and G. Vandebroeck. On the significance of the polar spot in ripe unfertilized and in fertilized ascidian eggs, 311.
Dawson, J. A., Walter R. Kessler and Joseph K. Silberstein. Mitosis in Amœba proteus, 125.
Decomposition, experimental, nitrogenous organic matter in sea water, 165.

Depressor substances, production by ultraviolet radiation of Limulus muscle, 75.

Dethier, Vincent G. Gustation and olfaction in lepidopterous larvæ, 7.

Digestion, enzymes of, in Daphnia and Polyphemus, Diaptomus and Calanus, 290.

Drosophila melanogaster, ovary transplants in, 47.

ELECTRICAL charge on nuclear constituents of salivary gland cells, 384.

Embryonic determination, production of intermediate-winged aphids and, 259.

Endomixis, induction of, in Paramecium aurelia, 196.

Enzymes, digestive, in Daphnia and Polyphemus, Diaptomus and Calanus, 290.

Evans, Gertrude. *See* Allee and Evans, 217.

Eyes, of Acanthephyridæ, 57.

FAURÉ-FREMIET, E. Licnophora lyngbycola, a new species of infusorian from Woods Hole, 212.

Feeding rate of Calanus finmarchicus in relation to environmental conditions, 233.

Food, effects on gastric epithelial cells of grasshopper, Melanoplus differentialis Thomas, 203.

——, rate of locomotion and reproduction in marine Amœba, 334.

Fox, Denis L., H. U. Sverdrup and John P. Cunningham. The rate of water propulsion by the California mussel, 417.

Fuller, John L. Feeding rate of Calanus finmarchicus in relation to environmental conditions, 233.

GASTRIC epithelial cells, effects of diet, in grasshopper, 203.

Gilchrist, Francis G. Budding and locomotion in the scyphistomas of Aurelia, 99.

Gonad, changes of, seasonal, in adult clam, 406.

——, primary, development in Venus mercenaria Linnæus, 389.

Gorgodera amplicava, development of, in final host, 80.

Grafflin, Allan L. Cyst formation in the glomerular tufts of certain fish kidneys, 247.

Grasshopper, effects of diet on gastric epithelial cells of, 203.

Gustation and olfaction in lepidopterous larvæ, 7.

Guttman, S. A. Evidence for the production of accelerator and depressor substances by ultraviolet radiation of Limulus muscle, 75.

HASLER, Arthur D. The physiology of digestion in plankton Crustacea. II. Further studies on the digestive enzymes of (A) Daphnia and Polyphemus. (B) Diaptomus and Calanus, 290.

Hodge, Charles, 4th. Some effects of diet on the gastric epithelial cells of the grasshopper, Melanoplus differentialis Thomas, 203.

Hopkins, Dwight Lucian. The relation between food, the rate of locomotion and reproduction in the marine Amœba, Flabellula mira, 334.

Hormone, chromatophorotropic, of Crustacea, 344.

Hydroid, new genus and method of asexual reproduction, 327.

Hyman, Libbie H. Reproductive system and copulation in Amphiscolops langerhansi (Turbellaria Acœla), 319.

ILYANASSA eggs, behavior of maturation spindles in polar fragments of, after centrifuging, 88.

Injury, mitotic rate in tadpole skin after, 37.

Intermediate-winged aphids and embryonic determination, 259.

KESSLER, Walter R. *See* Dawson, Kessler and Silberstein, 125.

Kidneys, fish, cyst formation in glomerular tufts of, 247.

Klein, Herbert M. *See* Churney and Klein, 384.

Kleinholz, L. H. Studies in the pigmentary system of Crustacea. I. Color changes and diurnal rhythm in Ligia baudiniana, 24.

——, ——. ——. Studies in the pigmentary system of Crustacea. II. Diurnal movements of the retinal pigments of Bermudan decapods, 176.

LICNOPHORA lyngbycola, new species of infusorian from Woods Hole, 212.
Ligia baudiniana, color changes and diurnal rhythm, 24.
Locomotion and budding in scyphistomas of Aurelia, 99.
——, rate of, reproduction, and food in marine Amœba, 334.
LOOSANOFF, VICTOR L. Development of the primary gonad in Venus mercenaria Linnæus, 389.
——, ——, —. Seasonal gonadal changes of adult clams, Venus mercenaria (L.), 406.
Loping of land-snails, 287.

MATURATION spindles, behavior of, in polar fragments of eggs of Ilyanassa obtained by centrifuging, 88.
MILES, SAMUEL STOCKTON. A new genus of hydroid and its method of asexual reproduction, 327.
Mitosis in Amœba proteus, 125.
——, rate of, in tadpole skin after repeated injury, 37.
Mnemiopsis, symmetry and regulation, 299.
MORGAN, T. H. The behavior of the maturation spindles in polar fragments of eggs of Ilyanassa obtained by centrifuging, 88.
Muscle, Limulus, production of accelerator and depressor substances by ultraviolet radiation, 75.

NITROGEN of particulate matter in the sea, 1.
Nitrogenous organic matter in sea water, experimental decomposition and regeneration, 165.
Nuclear constituents, electrical charge on, in salivary gland cells, 384.

ODLAUG, THERON O. Notes on the development of Gorgodera amplicava in the final host, 80.
Olfaction in lepidopterous larvæ, 7.
Onchidium, two types of respiration in, 41.
Ovary transplants in Drosophila melanogaster, 47.

PARAMECIUM aurelia, induction of endomixis, 196.
PARKER, G. H. The loping of land-snails, 287.
Phosphorus cycle and bacteria in the sea, 190.
Pigments, retinal, diurnal movements of, Bermudan decapods, 176.
Polar spot, significance of, in ripe unfertilized and in fertilized ascidian eggs, 311.

RAKESTRAW, NORRIS W. *See* von Brand, Rakestraw and Renn, 165.
Regulation and symmetry in Mnemiopsis leidyi, 299.
RENN, CHARLES E. Bacteria and the phosphorus cycle in the sea, 190.
——, —— —. *See* von Brand, Rakestraw and Renn, 165.
Reproduction, asexual, in new genus of hydroid, 327.
——, cycles of, and superfetation in poeciliid fishes, 145.
——, relation between food, locomotion rate and, in Amœba, 334.
——, system of, and copulation in Amphiscolops langerhansi, 319.
Respiration, two types, in Onchidium, 41.
Rhythm, diurnal, and color changes in Ligia baudiniana, 24.

SALIVARY gland cells, electrical charge on nuclear constituents, 384.
SCHECHTER, VICTOR. Calcium reduction and the prolongation of life in the egg cells of Arbacia punctulata, 366.
Sea water, nitrogenous organic matter in, experimental decomposition and regeneration of, 165.
SHULL, A. FRANKLIN. The production of intermediate-winged aphids with special reference to the problem of embryonic determination, 259.
SILBERSTEIN, JOSEPH K. *See* Dawson, Kessler and Silberstein, 125.
Snails, land, loping of, 287.
SONNEBORN, T. M. Induction of endomixis in Paramecium aurelia, 196.
Superfetation and reproductive cycle in poeciliid fishes, 145.
SVERDRUP, H. U. *See* Fox, Sverdrup and Cunningham, 417.

Symmetry and regulation in Mnemiopsis leidyi, 299.

TURNER, C. L. Reproductive cycles and superfetation in poeciliid fishes, 145.

ULTRAVIOLET light, effect on early development in eggs of Urechis caupo, 377.
—— radiation, production of accelerator and depressor substances, in Limulus muscle, 75.
Urechis caupo, eggs of, effect of ultraviolet light, 377.

VANDEBROECK, G. *See* Dalcq and Vandebroeck, 311.

WATER propulsion, rate of, by California mussel, 417.
WELSH, J. H., AND F. A. CHACE, JR. Eyes of deep sea crustaceans. I. Acanthephyridæ, 57.

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CONTENTS

Page

VON BRAND, THEODOR

Observations upon the Nitrogen of the Particulate Matter in the Sea 1

DETHIER, VINCENT G.

Gustation and Olfaction in Lepidopterous Larvae 7

KLEINHOLZ, L. H.

Studies in the Pigmentary System of Crustacea. I. Color changes and diurnal rhythm in Ligia baudiniana 24

CAMERON, JOHN ANDREW

The Mitotic Rate in Tadpole Skin after Repeated Injury 37

AREY, LESLIE B.

Observations on Two Types of Respiration in Onchidium . . . 41

CLANCY, C. W., AND G. W. BEADLE

Ovary Transplants in Drosophila melanogaster: Studies of the Characters Singed, Fused, and Female-Sterile 47

WELSH, J. H., AND F. A. CHACE, JR.

Eyes of Deep Sea Crustaceans. I. Acanthephyridae 57

GUTTMAN, S. A.

Evidence for the Production of Accelerator and Depressor Substances by Ultraviolet Radiation of Limulus Muscle 75

ODLAUG, THERON O.

Notes on the Development of Gorgodera amplicava in the Final Host 80

MORGAN, T. H.

The Behavior of the Maturation Spindles in Polar Fragments of Eggs of Ilyanassa Obtained by Centrifuging 88

GILCHRIST, FRANCIS G.

Budding and Locomotion in the Scyphistomas of Aurelia 99

DAWSON, J. A., WALTER R. KESSLER AND JOSEPH K. SILBERSTEIN

Mitosis in Amoeba proteus 125

Volume LXXII Number 2

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CONTENTS

Page

TURNER, C. L.

Reproductive Cycles and Superfetation in Poeciliïd Fishes . . 145

VON BRAND, THEODOR, NORRIS W. RAKESTRAW AND CHARLES E. RENN

The Experimental Decomposition and Regeneration of Nitrogenous Organic Matter in Sea Water 165

KLEINHOLZ, L. H.

Studies in the Pigmentary System of Crustacea. II. Diurnal movements of the retinal pigments of Bermudan decapods . . 176

RENN, CHARLES E.

Bacteria and the Phosphorus Cycle in the Sea 190

SONNEBORN, T. M.

Induction of Endomixis in Paramecium aurelia 196

HODGE, CHARLES, 4TH

Some Effects of Diet on the Gastric Epithelial Cells of the Grasshopper, Melanoplus differentialis Thomas 203

FAURÉ-FREMIET, E.

Licnophora lyngbycola, a New Species of Infusorian from Woods Hole 212

ALLEE, W. C., AND GERTRUDE EVANS

Some Effects of Numbers Present on the Rate of Cleavage and Early Development in Arbacia 217

FULLER, JOHN L.

Feeding Rate of Calanus finmarchicus in relation to Environmental Conditions 233

GRAFFLIN, ALLAN L.

Cyst Formation in the Glomerular Tufts of Certain Fish Kidneys 247

Volume LXXII Number 3

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BIOLOGICAL BULLETIN

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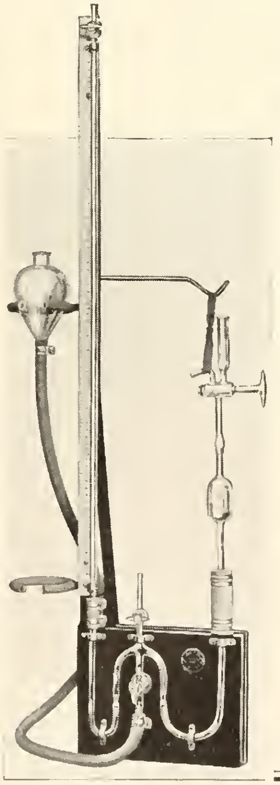

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CONTENTS

Page

SHULL, A. FRANKLIN
The Production of Intermediate-winged Aphids with Special Reference to the Problem of Embryonic Determination.... 259

PARKER, G. H.
The Loping of Land-snails.............................. 287

HASLER, ARTHUR D.
The Physiology of Digestion in Plankton Crustacea. II. Further studies on the digestive enzymes of (A) Daphnia and Polyphemus. (B) Diaptomus and Calanus........... 290

COONFIELD, B. R.
Symmetry and Regulation in Mnemiopsis leidyi, Agassiz.... 299

DALCQ, A., AND G. VANDEBROECK
On the Significance of the Polar Spot in Ripe Unfertilized and in Fertilized Ascidian Eggs.............................. 311

HYMAN, LIBBIE H.
Reproductive System and Copulation in Amphiscolops langerhansi (Turbellaria Acoela).............................. 319

MILES, SAMUEL STOCKTON
A New Genus of Hydroid and its Method of Asexual Reproduction.............................. 327

HOPKINS, DWIGHT LUCIAN
The Relation between Food, the Rate of Locomotion and Reproduction in the Marine Amoeba, Flabellula mira........ 334

ABRAMOWITZ, ALEXANDER A.
The Chromatophorotropic Hormone of the Crustacea 344

SCHECHTER, VICTOR
Calcium Reduction and the Prolongation of Life in the Egg Cells of Arbacia punctulata.............................. 366

CHASE, HYMAN Y.
The Effect of Ultra-violet Light upon Early Development in Eggs of Urechis caupo.............................. 377

CHURNEY, LEON AND HERBERT M. KLEIN
The Electrical Charge on Nuclear Constituents (Salivary Gland Cells of Sciara coprophila)....................... 384

LOOSANOFF, VICTOR L.
Development of the Primary Gonad and Sexual Phases in Venus mercenaria Linnaeus 389

LOOSANOFF, VICTOR L.
Seasonal Gonadal Changes of Adult Clams, Venus mercenaria (L.).............................. 406

FOX, DENIS L., H. U. SVERDRUP AND JOHN P. CUNNINGHAM
The Rate of Water Propulsion by the California Mussel.... 417

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